Yersinia pseudotuberculosis Membrane Vesicles Deliver Virulence Factors into Host Cells *In vitro*

Ajay Kalanjana Monnappa

School of Life Sciences
(Department of Biological Sciences)
Graduate School of UNIST

2016

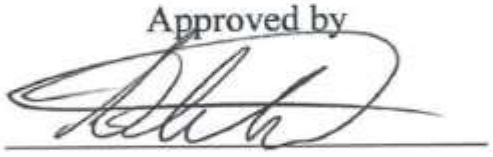
Yersinia pseudotuberculosis Membrane Vesicles Deliver Virulence Factors into Host Cells *In vitro*

A thesis submitted to the Graduate School of UNIST in partial fulfillment of the requirements for the degree of Combined- Masters of science and Doctor of Philosophy

Ajay Kalanjana Monnappa

11. 20. 2015

Approved by



Advisor
Prof. Robert J. Mitchell

Yersinia pseudotuberculosis Membrane Vesicles Deliver Virulence Factors into Host Cells *In vitro*

Ajay Kalanjana Monnappa

This certifies that the thesis of Ajay Kalanjana Monnappa is approved.

November 20, 2015

Advisor: Prof. Robert J. Mitchell

Prof. Hyung Joon Cha

Prof. Sang Sun Yoon

Prof. Nam Dougu

Prof. Cheol-Min Ghim

Yersinia pseudotuberculosis Membrane Vesicles Deliver Virulence Factors into Host Cells *In vitro*

Abstract

Yersinia pseudotuberculosis is a Gram-negative pathogenic bacterium that is believed to be an evolutionary ancestor of *Yersinia pestis*, a deadly pathogen causing bubonic plague. As a pathogen, *Y. pseudotuberculosis* causes a foodborne disease in humans and zoonotic infections in a variety of animal hosts, both wild and domestic. It is known that *Yersinia pseudotuberculosis* delivers various virulence factors into the host cells via type III secretion system (TTSS) during infection. Recent studies related to pathogenic bacteria showed most pathogenic bacteria uses an alternative mode of the delivery vehicle called membrane vesicles (MVs) in addition to their secretion systems. The current study focused on *Yersinia pseudotuberculosis* membrane vesicles and their impact on mammalian host cell *in vitro.* Transmission electron and super resolution microscopy analysis revealed 24 hours grown *Y. pseudotuberculosis* produced MVs. Further proteomic analysis by LC-MS/MS identified 887 proteins associated with membrane vesicles. MVs contained some secreted bacterial proteins, including 60kDa chaperonin (*GroEL*), Attachment invasion locus proteins (AilA), urease, etc. Based on subcellular localization predictions, MVs were found to be a double membrane structure. Further studies on human airways epithelial cell NuLi1 revealed purified MVs induced morphological changes and were shown to be cytotoxic as they were internalized into the cells successfully delivering 60 kDa Chaperonin and cytotoxic necrotizing factor (CNFy). When purified MVs were tested on murine macrophage, RAW 264.7 cells severer actin rearrangement could be observed within 3 hours. Significant loss in raw cell viability could be observed after 48 hours in a dose dependent manner as determined by MTT assay while confocal microscopy revealed giant cell multinucleated phenotype due to MVs exposure. Flow cytometric analysis confirmed that apoptosis is not a major event in cytotoxicity with only 2% cells found to be apoptotic. Furthermore, MVs induced high levels of TNF-α in raw cells. Finally, as cytotoxic necrotizing factor toxin (CNFy) is one of the crucial virulence factor allied with *Yersinia pseudotuberculosis* YPIII (+), we also tried to characterize this CNFy in detail in this study. Some early proteomics study confirmed that there was some signature post translational modification (PTM) of the *Y. pseudotuberculosis* CNFy protein. For further confirmation, we cloned and expressed *Y. pseudotuberculosis* (termed YCNFy) in *E.coli* (termed ECNFy). Analyzes with both *Yersinia* and *E.coli* CNFy for multinucleation in NuLi1 cells confirmed that YCNFy was 8 times more active than ECNFy. Comparative proteomics unveiled a greater PTM value of 61 in the *Yersinia* CNFy compared to that expressed in *E. coli* with a modification of only 6 amino acids. Out of all, only Lysine 1003 is "methylation" was found to be common in both YCNFy and ECNFy whereas 17 glutamic acid

methylation could only be found in YCNFy suggesting that PTM is required for the activity of CNFy. This study thus describes some novel and stirring characteristics of *Y. pseudotuberculosis* MVs and CNFy. Follow-up studies designed to utilize such unique findings should extend the scope of *Y. pseudotuberculosis* research regarding infection prevention and control.

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Host-microbe interaction and pathogenesis

Host-microbe interactions are as assorted as the organisms involved. They can be accidental or obligatory, temporary or persistent, and they can involve elusive or intense host response. But the outcome of the host is simple: health or disease. There are three well-known interactions between the host and microbe at the anatomical level. 1) Mutualism: in this, the microorganism and the host are equally benefited. For example, cattle and the rumen microflora. 2) Commensalism: in this kind of interaction, either the host or the microorganism experiences benefits while the other is not affected. Normal flora inhabiting in the eyes can be an example of this type of interaction. 3) Parasitism, also known as antagonism: in parasitism, one organism benefits at the expense of the other. For example, parasites use the human or animal gastrointestinal tract as an environment to colonize and reproduce. Pathogenic microbes come under this category. However, any microorganism considered a pathogen should be follows Koch's postulate[1]. But pathogenesis doesn't always end with disease manifestation. Pathogens cause diseases in selective hosts, and pathogens are distinguished from one another by their expressions of virulence factors. The interaction between pathogen and host is required for the disease progression[2]; however, not all host-pathogen interactions result in disease[3]. More recent views, originating primarily from studies of bacterial virulence, continued to focus on the ability of a pathogen to cause disease. The virulence property of a microbe has been used to describe a microbial characteristic[4].

Pathogenic bacteria have both virulence and immune evasion strategies, which may involve hiding from the immune system, interfering with the function of the immune system, and destroying elements of the immune system. Virulence comprises the use of various mechanisms to kill or cause the malfunction of host cells[5]. Various bacterial components interact with the host system (**Fig.1.1)**. Namely, capsules, which protect pathogens from phagocytosis, are a natural defense mechanism of the host whereby pathogenic microbes might be engulfed and killed by host immune cells[6]. Additionally, lipopolysaccharide (LPS) and outer membrane components may lead to septic shock,[7] and toxins can serve to damage host cells.[8] Most pathogens are intracellular, and some do not necessarily invade the host cell but use various secretion processes to deliver toxins and other virulence factors to the host cell to help the invasion as well as proteins to facilitate attachment to the host cell[9]. Once a pathogen is attached to a host surface, it invades host tissues[9] and may travel further inside them by expressing and secreting enzymes to digest host extracellular matrix proteins and polysaccharides.[10]

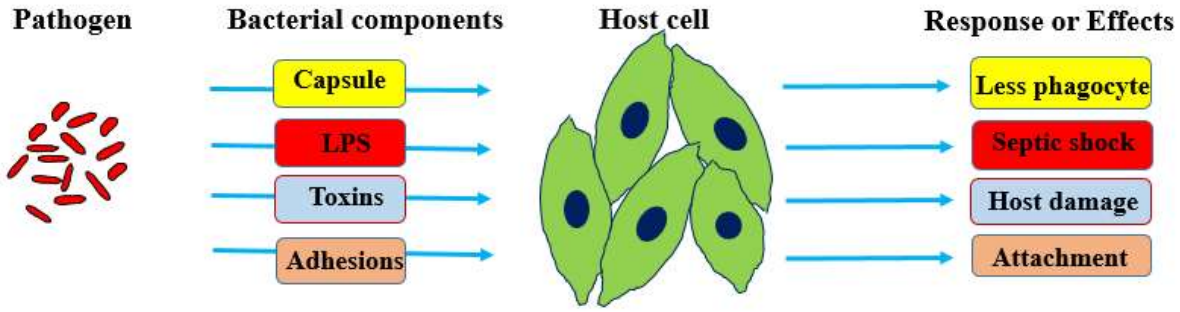


Fig 1.1 Various bacterial components interact with the host system.

1.2 The delivery of virulence factors via secretion systems

Bacteria are equipped with an inclusive variety of highly specialized mechanisms that secrete a range of molecules, including proteins and DNA[11]. Secretion systems are an important aspect of gram-negative bacteria and offer distinct advantages over the cell-associated material. Use of secretion systems to deliver bacterial factors to the host cell is a common strategy whereby pathogens induce uptake. Secreted molecules have significant roles in the response of a bacterium to environmental factors and several pathogenic events, such as adhesion, adaptation, and growth[12]. Based on the secretion system, the secreted substrates have three possible destinies: they remain allied with the bacterial outer membrane, they are injected into a target cell, or they are released into the extracellular space. Six secretion systems (Types I to VI) by which bacteria deliver virulence factors to the host system have been identified. Secreted material is typically considered to be soluble; however, prokaryotes and eukaryotes have specific mechanisms to secrete insoluble molecules. A study has revealed that gram-negative bacterial membrane vesicles (MVs), insoluble secretion pathways released from the envelopes of growing bacteria, serve as secretory vehicles for various cellular components of those bacteria.

1.3 Membrane vesicles (MVs)

Gram-negative bacteria have a specialized architecture of cell envelopes which split into two membranes known as the inner and outer membranes [13]. The peptidoglycan layer and the periplasmic space differ in lipid and protein composition. In most gram-negative bacteria, the outer membrane is composed mainly of LPS and phospholipids[14]. Between two membranes there is a viscous periplasmic space of approximately 13 nm which comprises approximately 7 to 40 percent of the total cell volume[15]. The cell envelop contains a large number of proteins which assist in several biological processes, such as nutrient acquisition, adherence, secretion, signaling, and protection from the environment[16]. Any stress to the envelope is lethal to the bacterium. To overcome environmental stress, gram-negative bacteria have evolved multiple mechanisms to monitor and repair damage caused by envelope stressors.

Gram-negative bacteria release membrane vesicles and periplasm through the production of MVs, small spherical structures 20 to 250 nm in diameter[17]. The existence of membrane vesicles first came to light when a cell-free filtrate of *Vibrio cholerae* was able to elicit an immune response in rabbits. As the outer membrane components of these bacteria are highly immunogenic, it was suspected that the components of the outer membrane (OM) were somehow leaching into the medium[18]. Later, it was shown that *E. coli* cultures grown on a lysine-limiting medium secreted LPS in the form of spherical structures which later released spherical membrane-bound vesicles[19], which was produced by pinching off the OM in *V. cholera* [20]. Similar studies have revealed the chemical composition of MVs present in outer membrane components and other cellular compartments. These structures have been variously termed 'membrane vesicles', 'extracellular vesicles', and 'outer membrane fragments'. The term 'outer membrane vesicles' is regularly used today[21].

MVs are produced when small portions of the OM bulge away from the cell, pinch off, and release. Soluble proteins are associated with MVs as entrapped periplasm and as externally adherent material. Secreted MVs can disseminate far from the cell and impart [22]. All types of gram-negative bacteria have been seen to produce MVs in a variety of environments, including in planktonic cultures, in fresh and salt water, in biofilms, and inside eukaryotic cells.[22] Within mammalian hosts, MVs play a crucial role in the interaction of bacteria with their environment and have been found to mediate and promote diverse functions, including pathogenesis, quorum signaling, nutrient acquisition, horizontal gene transfer, survival during stress, regulation of bacteria–bacteria interaction, and antibiotic resistance[23].

MV production is a common mechanism, and gram-negative MV formation has presumably evolved for a reason. Many studies of MVs have been directed at determining MV composition and functions[24]. Although several mechanisms for vesiculation have been proposed, it is presumed that vesiculation occurs by a well-conserved mechanism[21]; however, different types of MVs may exist and originate from different mechanisms. Also, subpopulations of MVs with distinct compositions may exist within a bacterial culture although MV population heterogeneity is a facet of the field that has yet to be explored in much depth[21]. Importantly, the field has suffered from scepticism, often as a result of initially unreliable terminology, as vesicles resulting from bacterial lysis were not differentiated from intact MVs for careful experimentation on MVs production and cargo and the mechanisms regulating these processes.

All gram-negative bacteria release MVs in every stage of growth and a range of different environmental conditions.[21] MVs aid bacteria in the secretion of soluble and insoluble cellular materials,[21] and although they contain many of the cellular components found within the parent bacterium, they are non replicative.[25] Previous analyzes of MVs from various pathogenic bacteria have

suggested that they contain LPS, peptidoglycan, periplasmic and membrane-bound proteins,[26] toxins,[27] DNA,[28] and RNA.[29] The key event of outer biogenesis includes outward protuberance of areas lacking membrane–peptidoglycan bonds. As well, membrane vesicle production is enhanced without a loss of outer membrane integrity, inclusion or exclusion of certain proteins and lipids, or membrane pilfering without direct investment of energy from ATP/GTP hydrolysis.[21] Secreted as insoluble and soluble bacterial envelope components, MVs are poised to play innumerable biological roles. An important feature of MVs is that the proteins associated with them exhibit biological activities. MVs act as delivery vehicles, as nucleates in the formation of bacterial communities (biofilms), and as contributors to bacterial survival and virulence. Nonvesiculated mutants have not been identified so far, but many genes whose alterations change have been identified.

1.4 Biogenesis of membrane vesicles (MVs)

Biogenesis of vesicles is regulated and does not result from or cause fractures in the outer membrane. It involves the packaging of proteins and lipids without the use of adenosine triphosphate (ATP) or other energy sources directly at the site of budding[,31]. In sum, vesiculation likely results from non exclusive events that depend on physiological circumstances or species. A series of events occurs during vesicle biogenesis, the first of which is the un budding of gram-negative envelopes. In this event, envelope proteins, including OM–peptidoglycan linking proteins, are distributed. The first step toward creating an MV is outward bulging of the outer membrane[30]. This event implies that buds form in areas where proteins linking the OM to the peptidoglycan layer are absent, which could occur if the peptidoglycan is disrupted, such as with antibiotics or autolysins. This could result in the release of MVs containing peptidoglycan fragments and portions of OM–peptidoglycan bridging proteins. Although this model accounts for increased vesiculation during envelope disruption, it is not likely the main mode of vesiculation.

Vesiculation can be increased by over a hundredfold without affecting membrane integrity[31], a feat hard to imagine if the peptidoglycan layer were heavily degraded. Other envelope events could trigger MVS budding. For example, OM–peptidoglycan links can be removed via target disruption. Evidence exists to support both models. As the linking protein OmpA is depleted but present in MVs and is excluded, simply relocating the links would provide the benefit of maintaining membrane integrity. Peptidoglycan links are present even if vesiculation is highly upregulated.

However, simply removing OM–peptidoglycan links does not suffice for the mechanism of MV formation because it does not consider that overexpressed and misfolded periplasmic proteins can cause budding and that specific proteins are enriched or excluded in MVs[30]. Some proteins concentrate at the inner leaflet of the outer membrane, thereby creating stress on the membrane, but budding occurs only after peptidoglycan linkages are removed from the outer membrane. This would increase the odds of a

bud forming when the links were removed and would allow vesiculation to be upregulated by the accumulation of periplasmic material as well as by the removal of OM–peptidoglycan linking proteins. Specific gathering of particular proteins at these sites would enrich these proteins in the MVs[21]. Alternative to or in addition to periplasmic turgor pressure, curvature-inducing molecules could cause membrane bulging. Localized concentration of curvature-inducing molecules leads to breakage of the underlying OM–peptidoglycan links after those membranes bud off. This event leads to enrichment of these molecules in MVs, as observed with molecules of *P. aeruginosa*[32]. Additionally, molecules which induce curvature will interact with specific periplasmic proteins and cause enrichment of particular MV cargo. In each situation, vesicle production is regulated by the accumulation and diffusion of envelope proteins. It is often proposed that MVs released from the cell when the bud grows to the point at which membrane curvature forces separation[33]. However, it is not clear that this happens. MVs are found in a variety of sizes, indicating that no critical threshold of curvature precipitates their release.

1.5 Factors influencing vesicle production

MV production is distinct from systems secreting soluble proteins. In MV secretion, the soluble material is released in a complex with or surrounded by insoluble material. Various environmental and physiological changes lead to the production of vesicles, for example, temperature, nutrient availability (low or high), vesiculation, and growth conditions. Cells growing at an exponential rate produce more MVs, and septation during cell division is one of the critical stages of MV production, possibly because of increased peptidoglycan turnover during cell division.[30]

1.6 Virulence factors associated with membrane vesicles

Various reports on membrane vesicles suggest that they are associated with virulent proteins.[32] These virulence factors include stress-related proteins, toxin adhesion, and other enzymes as well as known protein antigens such as LPS (Table 1). Membrane vesicles can act as a delivery vehicle for virulence factors by communicating with both prokaryotic and eukaryotic cells.[22] Recently, Maldonado et al. have demonstrated the hemolytic and leukotoxic activity of MVs internalized by human osteoblasts and synovial cells.[34] These cells produce increased levels of human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6).[34] A recent report has shown an association between MVs and invasion-related proteins in *Shigella flexneri*.[35] Recombinant attachment invasion locus proteins forming *Yersinia entercolitica* expressed in *E. coli* vesicles show a higher degree of invasion compared to non-recombinant ones.[36] This suggests that the attachment-invasion locus (Ail) protein is associated with membrane vesicles and contributes to the internalization of membrane vesicles. MVSs also transport a variety of ions, quorum-sensing signals, and metabolites.

Table 1. Toxins associated with membrane vesicles

SL no.	Bacteria	Toxin	Reference
1	*A. actinomycetemcomitans*	Cytolethal distending toxin	[37]
2	*Escherichia coli O157:H7*	Shiga toxin	[38]
3	*Vibrio cholera*	Cholera toxin	[20]
4	*Uropathogenic E.coli*	Cytotoxic Necrotizing Factor 1	[27]
5	*Helicobacter pylori*	Vacuolating toxin (VacA)	[39]
6	*Escherichia coli*	ClyA cytotoxin	[40]
7	*Kingella kingae*	RTX (repeat-in-toxin) toxin	[34]
8	*Bordetella pertussis*	Pertussis toxin	[41]
9	*Photorhabdus luminescens*	Toxin AB	[42]
10	*Actinobacil pleuropneumoniae*	Apx toxin	[43]

Table 1.2. Virulence factors associated with native membrane vesicles

SL no.	Species	Vesicle-associated virulence factor	Reference
1	*Shigella flexneri*	IpaB, IpaC, IpaD	[44]
2	*Actinobacillus pleuropneumoniae*	proteases	[43]
3	*Bacteroides fragilis*	Hemagglutinin	[45]
4	*Bacteroides succinogenes*	Cellulase, xylanase	[46]
5	*Photorhabdus luminescens*	Toxin AB, GroEL	[42]
6	*Pseudomonas aeruginosa*	Phospholipase C,	[47]
7	*Salmonella enterica*	Protective antigens	[48]
8	*Shigella flexneri*	IpaB, IpaC, IpaD (Invasion)	[49]
9	*Treponema denticola*	Dentilysin, adhesins, proteases	[50]
10	*Vibrio anguillarum*	Metalloprotease, hemolysin, phospholipase	[51]

1.7 Advantage of membrane vesicles over bacterial secretion systems

Membrane vesicles offer distinctive advantages over other bacterial secretion systems, as they can protect virulence determinants from host proteases and concentrate them for host cell delivery.[52] In addition, MVs can simultaneously deliver multiple virulence factors, MV-associated adhesions can provide tissue-tropic delivery of MV content,[53] and MVs can confer antibiotic resistance.[23] It should be appreciated that other basic physiological functions of MVs (see above) and their association with biofilms also contribute to bacterial pathogenesis by increasing bacterial adaptation and survival in the hostile host environment.[54] Intriguingly, both harmful and beneficial effects have been linked to MV production by bacteria present in the host gut microbiota.[55] Protein and virulence factors in membrane vesicles are inaccessible to proteases or peptidases.[56] Membrane vesicles help drug-resistant bacteria survive during antibiotic treatment and improve the biofilm formation abilities of pathogens.[23] Recent reports have revealed that bacteria use vesicles as long-distance delivery vehicles. MVS antigens disperse to sites far from initial colonization, such as the urine, blood, and some organs of *Borrelia burgdorferi*–infected mice, dogs, and humans.[57]

1.8 The genus *Yersinia*

Yersinia sp. is a rod-shaped facultative anaerobic gram-negative bacteria belonging to the enterobacteriaceae family. Out of its eleven species, only three, namely *Yersinia pestis, Yersinia entercolitica,* and *Yersinia pseudotuberculosis*, are considered important human pathogens. *Yersinia pestis*, well known as the causative agent of plague, is found chiefly in rodents and their fleas.[58] Other animals might carry it as well, but 85 percent of infections are caused by flea bites.[59] *Yersinia entercolitica* is the more common *Yersinia* species associated with foodborne infections. It is also reported to cause inflammatory bowel disease. This pathogen is transmitted via the faecal–oral route, most often by eating or drinking contaminated food or water.[60] It can also spread by coming in contact with infected animals or people.[61] This organism is most commonly found in raw pork or pork products such as pig intestines.[62]

1.9 *Yersinia pseudotuberculosis* and its virulence

Yersinia pseudotuberculosis is found in various environments like water.[63] There are also reports of its distribution within wild and domestic animals.[64] *Yersinia pseudotuberculosis* causes acute gastroenteritis and mesenteric lymphadenitis.[65] Although *Y. pseudotuberculosis* infections are distributed worldwide, *Y. pseudotuberculosis* is mostly invasive in nature compared to other species of *Yersinia*. During the invasion, *Y. pseudotuberculosis* expresses invasin (INV), attachment invasion locus (AilA) proteins, and adhesion (YadA) proteins, all of which mediate tight adhesion and entry into mammalian cells through β-integrins.[66] It also harbours a 70 kb virulence plasmid known as *pYV*. This plasmid encodes the *Yersinia* outer proteins (Yops), which include YopE, YopH, YopJ, YopM, YopT,

YpkA, and YopK.[67] Upon immediate contact with a target host cell, these Yops are delivered to it via a mechanism involving the Type III Secretion System (T3SS).[68] Aside from these, the *Y. pseudotuberculosis* cytotoxic necrotizing factor (CNFy) has been identified within host cells during infection and even contributes to the activity of the Yops.[69] However, the mode of delivery for CNFy and several other *Y. pseudotuberculosis* proteins remains unclear.[69-70] To date, membrane vesicle production in *Yersinia pseudotuberculosis* has not been reported. Recently published studies have described how bacterial pathogens employ MVs as vehicles to transport virulence factors and deliver them to host cells.[71] As such, MVs represent a novel and potent bacterial secretion mechanism that bypasses the need for direct contact between the bacterium and the host cell,[21, 72] does not require typical secretion machinery such as the T3SS and is exhibited by diverse pathogens.[53, 73] Moreover, one can easily envision a scenario in which a pathogen that incorporates invasive factors in its outer membrane, such as the AilA protein of *Y. pseudotuberculosis*, uses its MVs as vehicles to deliver virulence factors to host cells. Consequently, the production of MVs by *Y. pseudotuberculosis* and their potential involvement in pathogenesis, particularly in the delivery of virulence factors, has been investigated within this study.

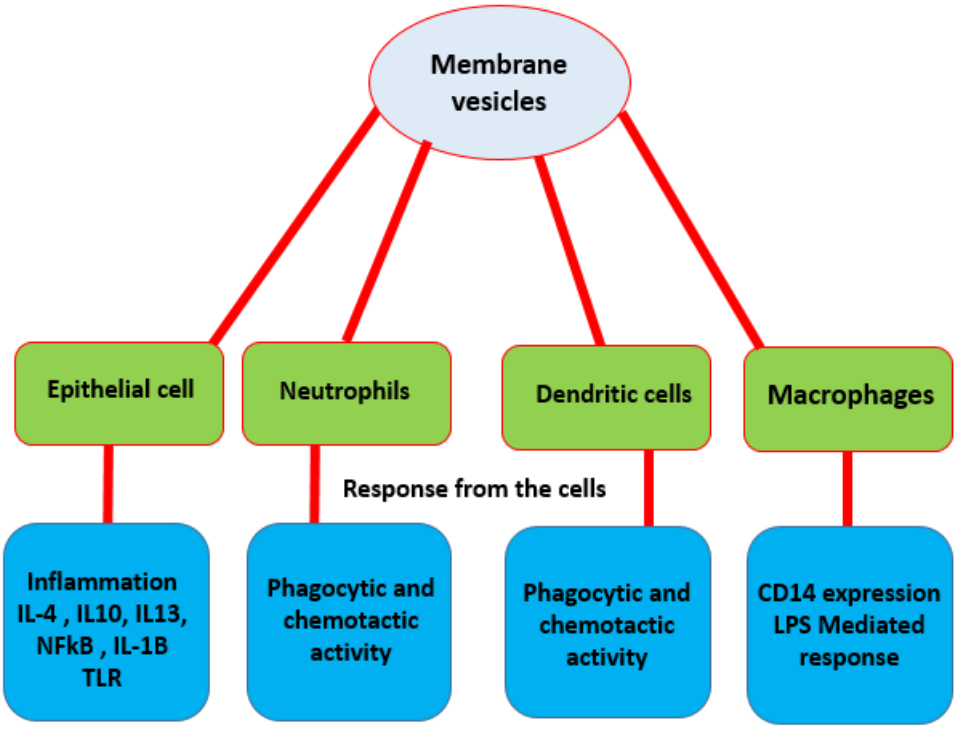


Fig.2 Response of host system to bacterial membrane vesicles

Chapter 2

Yersinia Pseudotuberculosis Membrane Vesicles Deliver Virulence Factor into Human Airway Epithelial Cell (NuLi-1)

2.1 Abstract

Membrane vesicles (MVs) are gaining much importance lately because of their bi-directional role in bacterial physiology and pathogenesis. Here we reported that *Yersinia pseudotuberculosis*, an important food-borne enteric pathogen produces membrane vesicles (MVs). 24 hour grown *Y. pseudotuberculosis* was associated with the production of MVs as revealed by transmission electron and super resolution microscopic analysis. Further characterization of the purified MVs were performed by SDS-PAGE, however, revealed significantly different protein profiles compared to proteins from stationary phase cells. Further proteomic analysis by LC-MS/MS, we identified 887 vesicular proteins with high confidence by three separate runs. Result revels vesicle contains a number of secreted bacterial proteins, including 60kDa chaperonin (*GroEL*), urease and attachment invasion locus proteins (AilA). Based on subcellular localization predictions, MVs composed of an outer membrane, periplasmic and cytoplasmic proteins. Further studies with human airways epithelial cells, purified MVs induced morphological changes in human airways epithelial cells and MVs were shown to be cytotoxic as they internalized by human airways epithelial cell.

2.2 Introduction

Yersinia pseudotuberculosis is a Gram-negative enteric pathogen that is believed to be a direct evolutionary ancestor of *Yersinia pestis*, the causative agent of the bubonic plague[74]. As a pathogen, *Y. pseudotuberculosis* causes foodborne diseases in humans and zoonotic infections in a variety of wild and domestic animal hosts [75]. This bacterium is transmitted via the fecal-oral route where, after entry into the host, it penetrates the intestinal epithelium by initially infecting Peyer's patches and spreads after that to the lymphatic system and the mesenteric lymph nodes[76]. In humans, *Y. pseudotuberculosis* is associated with several medical conditions, including the gastrointestinal disease yersiniosis [77], mesenteric lymphadenitis [78], fatal septicemia [79] and at times they may lead to post-infectious reactive arthritis [66a, 79-80].

Y. pseudotuberculosis has evolved an exquisite method for delivering virulence factors and effectors into cells of the host immune system where they inhibit signaling cascades and block the cells response to infection[81]. Moreover, adhesion molecules and a Type III secretion system are critical for the establishment and progression of the infection[82]. This pathogen expresses the invasin (INV), attachment invasion locus protein (AilA) and adhesion (YadA) proteins, all of which mediate tight adhesion and entry into mammalian cells through β-integrins [66], and also harbors a 70-kb virulence plasmid known as p*YV*. This plasmid encodes the *Yersinia* outer proteins (Yops), which include YopE, YopH, YopJ, YopM, YopT, YpkA and YopK[67a, 67b]. Upon intimate contact with a target host cell, these Yops are delivered into the host cell via a mechanism involving the Type III Secretion System (T3SS)[68]. Asides from these, the *Y. pseudotuberculosis* cytotoxic necrotizing factor (CNFy) has been identified within host cells during infection and even contributes to the activity of the Yop proteins[69]. However, the mode of delivery for CNFy and several other *Y. pseudotuberculosis* proteins in the milieu of host cells remain unclear[69-70].

Many Gram-negative bacteria, both pathogenic and non-pathogenic, have been shown to produce membrane vesicles (MVs)[71]. Although the exact reason for their production is not always clear, MVs can contain biologically active proteins and play various roles in diverse biological processes, including biofilm formation and the potential to critically impact disease progression[71]. For instance, recently publicized studies describe how bacterial pathogens employ MVs as vehicles to transport virulence factors and deliver them into host cells [83] [71]. As such, vesicles represent both a novel and potent bacterial secretion mechanism that by-passes the need for direct contact between the bacterium and the host cell [21, 72], does not require typical secretion machinery, including T3SS, and is exhibited by diverse pathogens [53, 73]. Moreover, one can easily envision a scenario where a pathogen that incorporates invasive factors in its outer membrane, such as the AilA protein of *Y. pseudotuberculosis*, uses its MVs as vehicles to deliver virulence factors into the host cells. Consequently, the production of MVs by *Y. pseudotuberculosis* and their potential involvement in pathogenesis, particularly in the delivery of

virulence factors, was investigated within this study. To the best of our knowledge, this is the first ever study reporting production and virulence association of MVs from *Y. pseudotuberculosis*.

2.3 Materials and Methods

2.3.1 Bacterial strain and growth condition

Yersinia pseudotuberculosis ATCC29833 obtained from KACC South Korea and *Y. pseudotuberculosis* YPIII with virulence plasmid (+) NR4380 obtained from Biodefense and emerging infections research resources repository (BEI Resources, USA) and *E. coli* NlpI obtained from Keio collections(Keio group, Japan). Strains were maintained as frozen 20% glycerol stocks at -80°C. Upon need, this strain was grown on Luria-Bertani agar plates (BD; Difco, USA) at 30°C overnight. From this plate, a single colony was inoculated into 5 ml of LB broth and grown overnight at 30°C with agitation (250 rpm) florescent *Yersinia* strains were developed by transferring pAmCyan plasmid (Promega, USA)

2.3.2 Culture of human airway epithelial cell line (NuLi-1 Cells)

Bronchial epithelial cell (NuLi-1) (ATCC CLR 40110) obtained from ATCC were cultured in T75 flasks in bronchial epithelial cell growth medium (BEGM; Lonza, USA) substituted with antibiotic Normocin, 100 µg/ml (Invivogen, USA), until they were confluent. Cells were then washed with sterile DPBS and trypsinized for 5 min to detach from the flask. Cells were then washed with complete medium, counted using hemocytometer and diluted appropriately to seed in an appropriate culture dish for the experiments.

2.3.3 Culture of human breast epithelial cell line (MCF-10A Cells)

Mammary gland/breast epithelial cells (MCF-10A) were cultured in T75 flasks in Dulbacco's Modified Eagle's Medium (DMEM-F12 1:1 with 15 mM HEPES; Gibco, USA). The media is substituted with 5% heat-inactivated fetal bovine serum (ATCC, USA), 20ng/ml of EGF, 10 µg/ml of insulin (Sigma-Aldrich, USA), and 100 µg/ml of Normocin until they were confluent. Then cells were washed with sterile DPBS and trypsinized 5 min detached from the flask. Cells were then washed with complete medium, counted and diluted appropriately to seed on the plate required for the experiment.

2.3.4 Cloning of 60kDa chaperonin and CNFy

60 kDa chaperonin from both *Y. pseudotuberculosis* ATCC 29833 and YPIII (+) and CNFy from *Y. pseudotuberculosis* YPIII(+) were amplified by PCR from genomic DNA using gene specific primers **(Table 2.6)** , thereby introducing restriction sites for restriction enzymes Xba1 and Xho1. The resulting PCR product was ligated into the expression vector pET31b+ and transformed into *E. coli* Dh5α. After confirmation of insert, the plasmid harboring CNFy or 60kDa chaperonin were transformed into *Y. pseudotuberculosis* and *E. coli* NlpI.

2.3.5 Purification of CNFy and 60kDa chaperonin

Y. pseudotuberculosis YPIII(+) harboring pET31B+ CNFy, *Y. pseudotuberculosis* ATCC 29833 Harboring 60kDa chaperonin both strains were grown in LB medium with antibiotic ampicillin 100μg/ml. Then incubated at 30°C until the bacteria reaches optical density of OD 1.0. Then cells were collected by centrifugation the resulted pellet were suspended in lysis buffer(50 mM Tris-HCl pH 7.5; 200 mM NaCl) then supplemented with lysozyme (1mg/ml). Suspension kept in ice for 30 minutes before sonication. After 30 min cells had been sonicated lysate were centrifuged to remove the cell debris. Further lysate were purified by affinity chromatography on Ni-NTA agarose (Qiagen; USA). loaded beads were washed with lysis buffer (with 20mM imidazole) at 4 °C. The CNFy and chaperonin were eluted from the beads (500mM imidazole with lysis buffer) twice for 10 min at room temperature

2.3.6 Determination of plasmid pET31b+ copy number in *Y. Pseudotuberculosis* by Real-time qPCR

To determine the copy number of plasmid we followed previously reported protocol with slight modifications[84]. *Yersinia pseudotuberculosis* ATCC 29833 and *Yersinia pseudotuberculosis* YPIII (+) harboring pET31b (+) plasmid were cultured in 15 ml of LB media 50 ml conical tube at 30°C and 37 °C. Total DNA was extracted from each of the cultures during the exponential growth phase 0.8 OD by periodic measurement of optical density (OD) at 600 nm. The extraction was performed using the DNA purification Kit (Cosmo, South Korea), the template DNA extracted from *Y. pseudotuberculosis* was normalized to 1ng/μl. The concentration of extracted DNA was measured using Nano drop (thermos scientific, USA). The prepared template DNA was analyzed to quantify bla and CNFy triplicate in real-time qPCR assay. Real-time qPCR amplification and analysis were performed using a Light cycler instrument with software version 3.5 (Roche Diagnostics). The threshold cycle (Cp) was determined with standard Cp value for the CNFy Which cloned in pET31b (+). The vector the copy number theoretically calculated. CNFy in both *Yersinia* was used as internal control in *Yersinia* has a single copy of CNFy.

2.3.7 Membrane vesicle isolation

Purification of the membrane vesicle is based on the previous protocol with slight modifications. Cultures of *Y. pseudotuberculosis* ATCC 29833 or YPIII (+) were grown in 500ml LB broth for 24 hours as described above. The cells were removed by pelleting (4000xg, 20 min) and then filtering the supernatants twice with 0.22-micron filtration units (Stericup; Millipore, USA). The MVs present in the supernatant were then concentrated using a 100-kDa tangential filtration concentration unit (Amicon

Ultra-15; Millipore USA) to approximately 5 ml. Subsequently, they were pelleted (40,000×*g*, 2 hours) and suspended in 1 ml of 25 mM phosphate buffered saline (PBS, pH 7.2). The concentration of MVs was quantified based on the protein content using the Bradford colorimetric assay method (Life Technologies, USA).

2.3.8 Quantification of MVs by FACS

Yersinia pseudotuberculosis ATCC 29833 and *E.coli NlpI* were used to determine the production of MVs by FACS. *E.coli NlpI* secretes a high number of MVs so initial FACS gate setting was done using purified *E.coli NlpI* MVs. To determine MVs production by *Y. pseudotuberculosis* ATCC 29833 bacteria were grown in LB media at 30°C in shaking incubator. The optical density was measured at different time points, the cell-free supernatant at each time point were collected by pelleting the bacteria by centrifuging at 5000 RPM 20 min followed by filtration through 0.22-micron filter. Collected cell free media were analysis by FACS, FSC gated population were recorded for 30 seconds for each sample MVs number calculated based on the flow rate of the FACS system.

2.3.9 Determination of toxicity of *Y. pseudotuberculosis* ATCC 29833 cell free media

It was reported that *Y. pseudotuberculosis spent* media was toxic to the mammalian cell. To confirm *Yersinia pseudotuberculosis,* ATCC29833 *were* grown in LB media at the aerobic and semi-aerobic condition for 24 hours. Spent media were collected, by removing bacteria by centrifugation at 5000 RPM for 20 min followed by 0.22 micron filtration. Toxicity of the cell free media on NuLi-1 cell were determined by MTT assay.

2.3.10 Observation of MVs using TEM and super-resolution microscopy (SR-SIM)

For electron microscopy, MVs were purified as mentioned above. 2µl of MVs/bacteria culture were placed on carbon-coated Formvar grids (Polysciences, Inc., USA). The samples were treated with 2.5% glutaraldehyde for 1 hour. Excess fixing agents were eliminated during the dehydration of samples as they were washed sequentially in a series of an aqueous solution containing increasing amounts of ethanol and finally in absolute acetone. The samples were then negatively stained with 0.1% uranyl acetate and examined under a transmission electron microscope (JEOL 1200 EX, USA) present in the UNIST central research facility (UCRF). To obtain the super-resolution microscopy image, concentrated MVs were labeled with rhodamine B-18 (Life Technologies, USA) for 20 min. After staining, unbound dye was removed by ultracentrifugation at 40000 rpm for 2 hours. Concentrated MVs were diluted in PBS. Approximately 5 µl (100 µg/ml) of MVs were visualized under a super-resolution microscope (ELYRA S.1 super-resolution structured illumination [SR-SIM]; Carl Zeiss, Germany) operated by Zen 2012 software.

2.3.11 Visualization of internalized MVs by confocal microscopy

For microscopy, a monolayer of human cells was prepared on 8 well-chambered cover glass (Nunc® Lab-Tek® II Thermo Scientific) by seeding $1X10^4$ human airway epithelial cells/well and incubated in a CO_2 incubator at 37ºC for 24 hours. Before MVs treatment, human airway epithelial cells were stained with CFSE by replacing media with 0.5ml of CFSE/CMTPX (Molecular probe: Invitrogen, USA) in PBS final concentration of 5µg/ml and incubated at 37ºC in a CO_2 incubator for 10 min. After incubation, cells were washed twice with PBS and added to 0.5 ml of BGEM media. Stained cells were incubated for 1 hour at 37ºC in a CO_2 incubator with 5% CO_2. For invasion assay, purified MVs stained with rhodamine BR-18/ as mentioned above stained MVs or MVs from CFP expressing *Yersinia* diluted to 10ug/ml in BGEM media. Initially, well was replaced with 0.5 ml of BGEM with MVs and without MVs as a control was incubated for 2 hours at 37ºC. After incubation, unbound MVs were removed and replaced with 0.5 ml of BGEM media. For time-lapse imaging, cultured cells were treated with purified vesicle from CFP expressing *Yersinia pseudotuberculosis* YPIII (+) strain and 20 µg/ml of vesicle were treated to each well. Cells were incubated for 2 hours. An unbound vesicle was removed after 2 hours and washed with DPBS 3 times; then cells were replenished with fresh complete media and imaged at different interval of using LSM 700 laser scanning confocal microscope (Carl Zeiss, Germany) operated by ZEN 2009.

2.3.12 Proteomic Analysis of *Y. pseudotuberculosis* Vesicles

To identify proteins associated with the outer membrane vesicle, purified vesicle preparations (12 µg) were applied to 12% Tris-glycine SDS-PAGE gel (Bio-Rad, USA). After staining with Coomassie blue, the each band separated into 6 were subjected to in-gel tryptic digestion, as described by Shevchenko and co-workers [85]. The resulting tryptic peptides were analyzed by LC-MS/MS. All mass analysis were performed on an LTQ-Orbitrap (Thermo, Bremen, Germany) equipped with a nanoelectrospray ion source. To separate the peptide mixture, we used a C18 reverse phase HPLC column (150mm x 75um i.d.) using an acetonitrile/0.1% formic acid gradient from 10 to 24% for 90min at a flow rate of 300 nl/min.

For MS/MS analysis the precursor ion scan MS spectra (m/z 400–2000) were acquired in the Orbitrap at a resolution of 60,000 at m/z 400 with an internal lock mass. The 20 most intensive ions were isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID).All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.1.14) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest and X! Tandem were set up to search the *Y. pseudotuberculosis* protein sequence database (9823 entries, UniProt (http://www.uniprot.org/)) assuming the digestion enzyme trypsin.Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM.

Carbamidomethyl of cysteine was specified in Sequest and X! Tandem as a fixed modification. Deamidated of asparagine and glutamine, methyl of glutamic acid, lysine, glutamine and arginine, oxidation of methionine, acetyl of lysine, serine, threonine and the n-terminus and phospho of serine, threoneine and tyrosine were specified in Sequest and X! Tandem as variable modifications. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus were specified in X! Tandem as variable modifications.

2.3.13 Criteria for protein identification

Scaffold (version Scaffold_4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [86]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins are annotated with GO terms from NCBI (downloaded Aug 1, 2014). [87]

2.3.14 Cell viability and cytotoxicity assays

The toxic effects of purified MVs in-vitro were analyzed by quantitatively determining cell viability of human airway epithelial (HAE) cell line, NuLi-1 (ATCC CLR 40110). In 96 well plate 50% confluent airway epithelial (HAE) was prepared and treated with 100ul of 10ug/ml of MVs in BGEM media added into each well and incubated 72 hours at CO2 incubator. Briefly, MTT solution (5 mg/ml) was added to each well in an amount equal to 10% of the culture medium volume. Cells are incubated for two h at 37°C; absorbance was measured at a wavelength of 540 nm, and the percentage of viable cells were calculated as the ratio of absorbance of stained cells.

2.3.15 Immunostaining of F-actin

To study the influence of the MVs on actin cytoskeleton rearrangements, a monolayer of NuLi-1 was incubated with purified MVs or PBS for 3 hours. Subsequently, cells were fixed with 4% paraformaldehyde (in PBS) overnight at 4ºC temperature after fixation cells were washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, USA) in PBS for 5 min. The actin cytoskeleton stained with phalloidin rhodamine (0.5 µg/ml PBS; Invitrogen, USA) for 1 hour at room temperature. Cells were washed with PBS and the nuclei stained with DAPI (1 µg/ml in PBS) for 5 min at room temperature. Cells were visualized using a laser confocal microscope (LSM 700 and the Zen program (Zeiss, Germany).

2.3.16 Western blotting

The samples of cellular protein from treated and untreated cell extracts separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then electro blotted onto a polyvinylidene difluoride (PVDF) membrane. The immunoblot was incubated for 2 hours with blocking solution (5% (wet/vol) skim milk Difco), followed by incubation for 2 hours with Anti His-tag antibody conjugated to HRP. Blots washed with Tween 20/Tris-buffered saline (TBST, 20 mM Tris-HCl buffer, pH 7.6), containing 137 mM NaCl and 0.05% (vol/vol) and images using LAS 200 System (GE Healthcare, USA).

2.3.17 Semi-quantitative analysis of the chaperonin in MVs

MVs were purified from both *Y.pseudotuberculosis* ATCC29833-Chap *and Y. pseudotuberculosis* YPIII(+)-Chep. Chaperonin were purified from *Yersinia pseudotuberculosis* ATCC 29833-Chap by affinity chromatography Using Ni-NTA agarose as mentioned previously, the purified chaperonin were quantified using Broadford assay. A known concentration of the chaperonin and MVs loaded to SDS page followed western blotting using anti-his tag HRP conjugate(Abcam, USA).

2.3.18 Quantification of CNFy by ELISA

For quantification 10 µg of MVs prepared by treating with or without 0.1% TritonX. A know the concentration of purified recombinant CNFy expressed in YPIII (+) used as a standard. Both standard and purified MVs were coated on costar 96 well plate using a coating buffer (0.1M sodium bicarbonate buffer; pH 9.6) incubated for 2 hours at room temperature then blocked by adding 200µL of blocking buffer (1% BSA in washing buffer to each well). Incubate for 30 minutes at 37°C. Wash plate 3 times with 200 µL of wash buffer. Add of HisProbe-HRP1:5000 dilutions. Put working solution to each well and incubate for 15 minutes at room temperature. Wash plate 4 times with 200µL of wash buffer. Add 50µl Color reagent A and B (R and D systems, USA) incubated for 30 min at room temperature, then reaction were stopped by adding solution (R and D Systems, USA) was added and allowed to develop color then measured absorbance at 450 nm.

2.19 Quantification of multinucleation

To determine the multinucleation, NuLi-1 cells (2000 cells/well) were seeded on grown 96 well-plate or 24 hours with 5% CO_2 then incubated with the different concentration of MVs for 48 hours. in a CO_2 incubator with 5% CO_2 were stained with CMTPX red then incubated with the. After incubation cells were washed with DPBS (Sigma-Aldrich, USA) and fixed with 4.0 % of paraformaldehyde for 10 min at room temperature. Fixative were removed by washing three times with DPBS and by washing, and fixed cells were counterstained with DAPI(5µg/ml(Invitrogen, USA) . Then cells were visualized under

the confocal microscope (LSM 700: Carl Zeiss, Germany) at the magnification 20X and images captured using Zen 2009 software.Multinucleated cells were visually counted and calculated percentage of multinucleated cells.

2.3.20 Confirmation of packaging of the 60 kDa Chaperonin

To determine the packaging of chaperonin the MVs form *Y. pseudotuberculosis ATCC 29833* was purified at different time point MVs concentration was determined by Bradford assay from each time point 15ug of MVs were analyzed by SDS-PAGE.Further confirmation to demonstrate chaperonin only abundant in the MVs it is not abundant in the whole cells MVs and whole cell from wild-type *Y. pseudotuberculosis* ATCC29833 and YPIII(+) and recombinant with *Y.pseudotuberculosis* ATCC 29833-Chap YPIII-Chap was prepared. Both whole cell and MVs samples were boiled with SDS-PAGE loading buffer then concentration of protein was determined by Broadford assay. 15ug of protein from each sample were loaded to SDS-PAGE for silver staining and by western blotting.

2.4 Result and discussion

2.4.1 *Yersinia pseudotuberculosis* Secretes membrane vesicles (MVs)

Filtered (0.22μm), cell-free spent media from cultures of *Yersinia pseudotuberculosis* ATCC 29833 grown overnight under several different conditions (**Fig 2.1)** was found to be harmful to NuLi-1 airway epithelial cells, reducing their viability (**Fig 2.2**). Although the degree of impact varied somewhat based on the growth conditions used for the pathogen, the dose-dependent nature of all the samples suggested that some effector(s) was present in the cell-free supernatants.

Recently, several groups demonstrated that various pathogenic bacteria produce and secrete membrane Membrane vesicles (MVs), but this was not verified for *Y. pseudotuberculosis*. Consequently, we imaged *Y. pseudotuberculosis* ATCC 29833 using transmission electron microscopy (TEM). As shown in **Fig 2.3)**, we found MVs clearly associated with and budding off of the bacterial cells. Subsequently, as cultures of *Y. pseudotuberculosis* ATCC grew, 0.22μm-filtered supernatant samples taken and the number of MVs within each was determined via FACS counting (**Fig 2.4**) after first labeling the MVs with Rhodamine B-R18, a membrane binding dye that generates a strong red fluorescent signal as shown in (**Fig 2.5**) The number of MVs within the cultures continually increased as *Y. pseudotuberculosis* ATCC grew but was more pronounced once the cultures entered the stationary phase. Although the sizes of the MVs in (**Fig 2.3)** and other TEM images varied greatly between approximately 20 nm to 400 nm in diameter, the sizes were more clearly visible after purification (**Fig2.6**).

2.4.2 *Y. pseudotuberculosis* MVs are Double MVs

In addition to FACS enumeration, labeling of the purified MVs with rhodamine B-R18 allowed us to visualize them using super-resolution confocal microscopy (**Fig 2.5)**. Unexpectedly, when MVs were purified from cyan fluorescent protein (CFP) expressing *Y. pseudotuberculosis* ATCC 29833 cultures, they fluoresced green (**Fig 2.5)**. The clear and widespread presence of CFP, a cytoplasmic protein, within the secreted MVs strongly implied that many of the *Y. pseudotuberculosis* ATCC29833 MVs that were purified are double-membrane. Another related and important idea that stemmed from this observation was that the MVs also harbored other cytoplasmic proteins and possibly known virulence factors. A comparative analysis of the proteins present within the whole cells and those in the purified MVs using SDS-PAGE gel showed they were quite distinct from one another (**Fig 2.7**). One clear example of this was the presence of a dominant 60 kDa band in the MVs preparation. Using mass spectrometry, a total of 879 proteins that spanned the entire stratum of the bacterium, from the cytoplasm to the outer membrane **(Tables 2.2 to 2.5),** were identified within the *Y. pseudotuberculosis* ATCC29833 MVs. The cytoplasmic fraction comprised the largest grouping, with 484 proteins (55%) (**Fig 2.8**). **Table 2.1** lists the top thirty proteins identified according to the number of hits, with cytoplasmic proteins occupying the top four spots. The large presence of cytoplasmic proteins within the *Y. pseudotuberculosis* MVs further affirms that they are a double membrane. Moreover, the two

most abundant proteins identified were virulence factors - the 60 kDa chaperonin and the alpha-subunit of urease, which has a size of 61 kDa. Combined, these two proteins constituted more than 83% of all hits seen in the LC-MS-MS analysis for the purified MVs, a finding that strongly supports the SDS-PAGE results presented in Fig **2.5.**

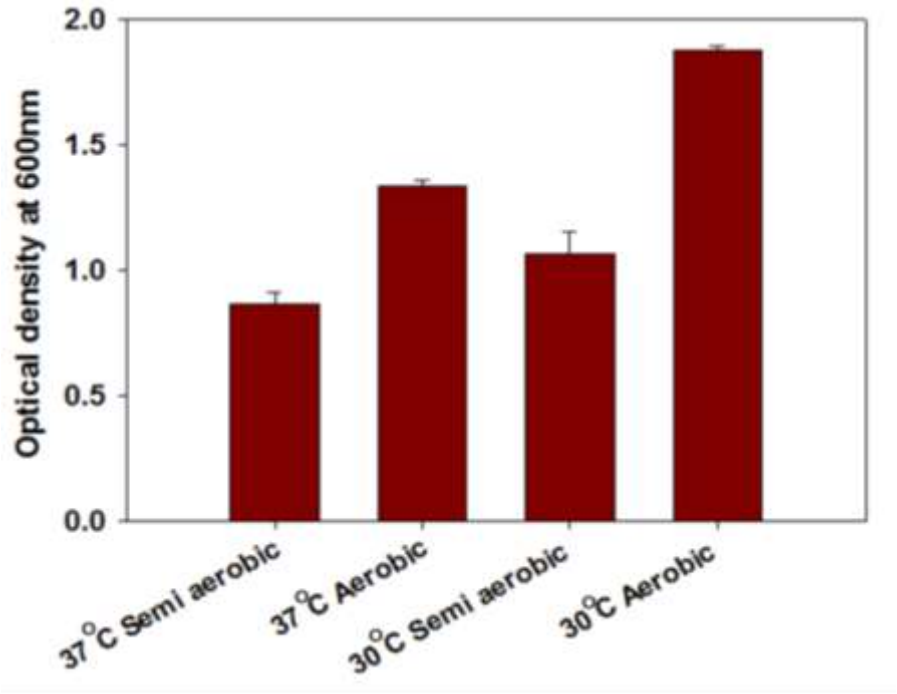


Fig 2.1 Growth of *Y. pseudotuberculosis* ATCC 29833 at different conditions. OD at 600nm was measured after 24 hours.

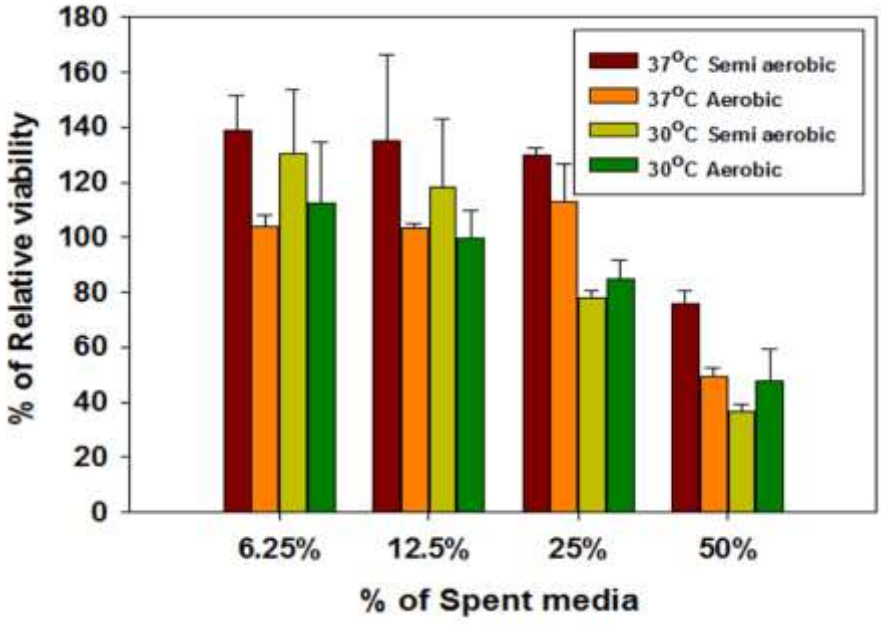


Fig 2.2 Viability of NuLi-1 cells after treatment of *Y. pseudotuberculosis* ATCC 29833 spent media. Cell-free supernatant from 24 hours grown *Y. pseudotuberculosis* at different growth condition was collected. Cell-free supernatant were treated on a monolayer of NuLi-1 cells then the viability of cells were determined by MTT assay after 48 hours of treatment.

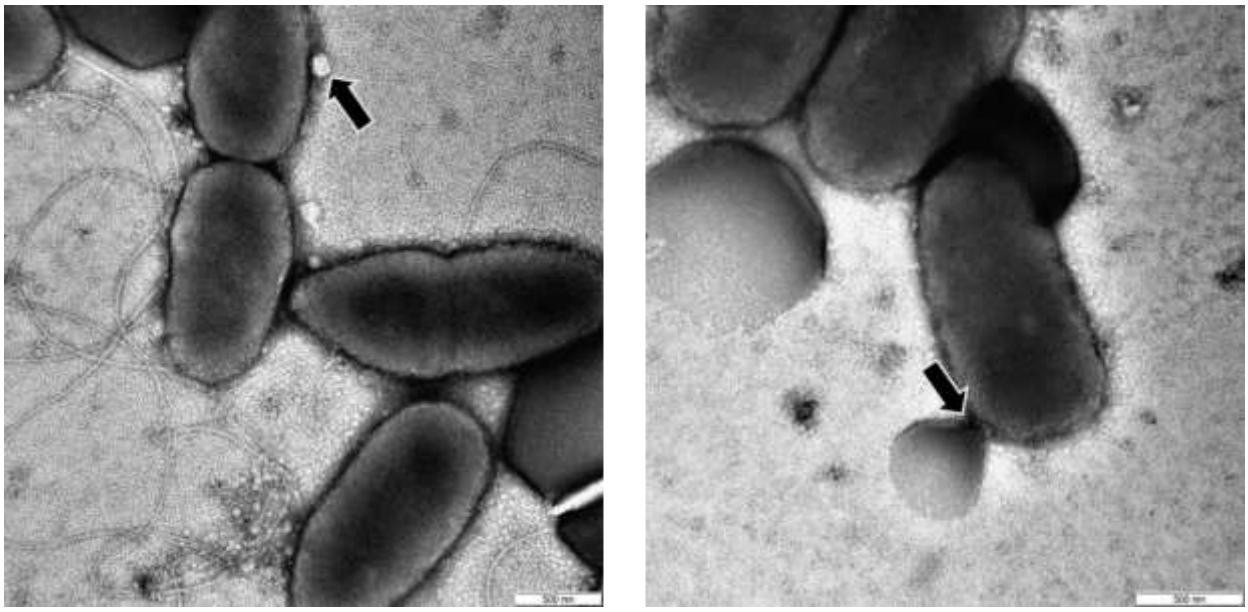


Fig 2.3 Transmission electron microscopy (TEM) image of *Y. pseudotuberculosis* ATCC29833 producing MVs. 24 hour grew *Y. pseudotuberculosis* ATCC29833 culture were subjected to TEM imaging. MVs are clearly associated with and budding off of the bacterial cells (Scale bar 500 nm)

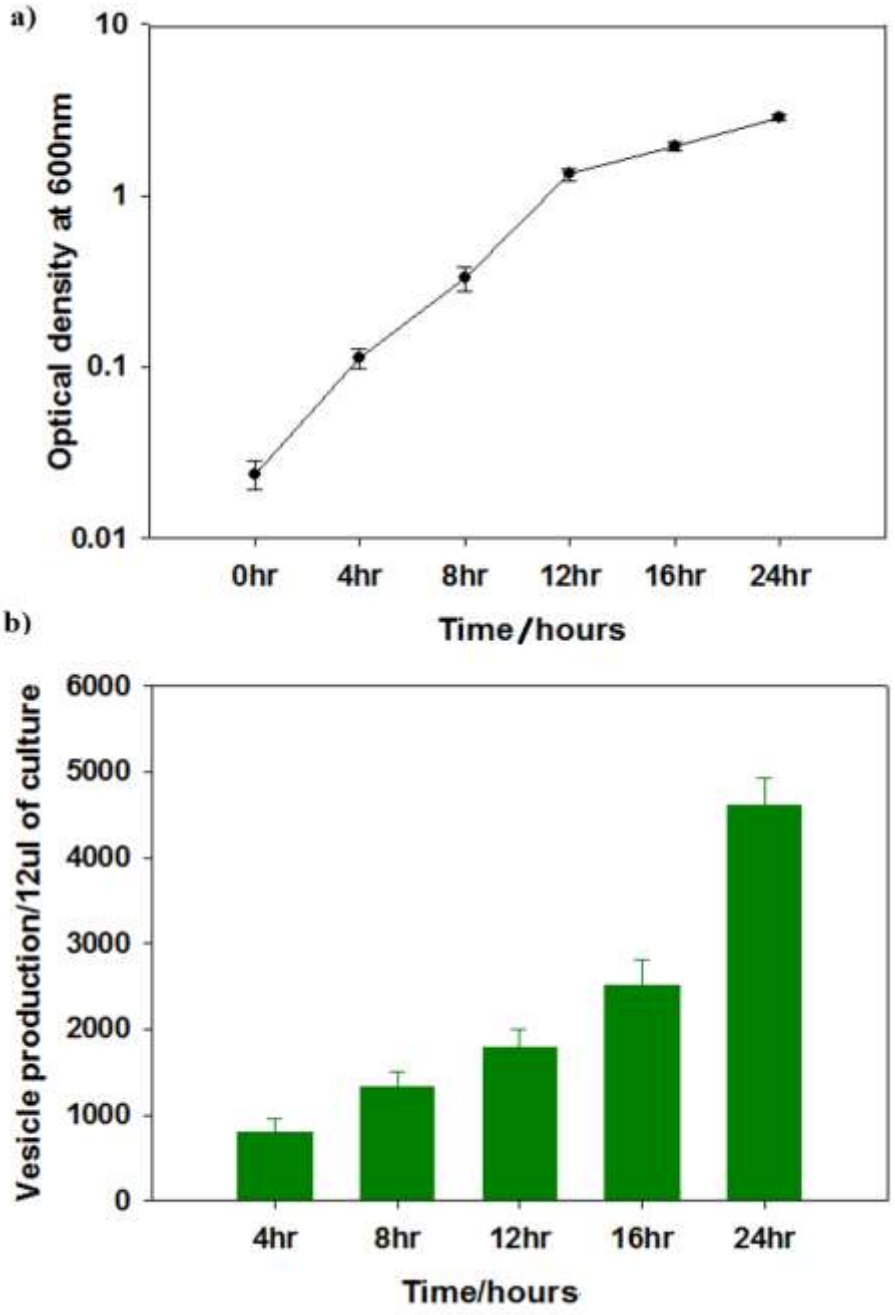


Fig 2.4 FACS analysis to quantify the MVs production within each time point/ growth stages.

a) OD of *Y. pseudotuberculosis* ATCC 29833 at different time points represented**. b)** Vesicle production by *Y. pseudotuberculosis* ATCC 29833 quantified by FACS analysis.

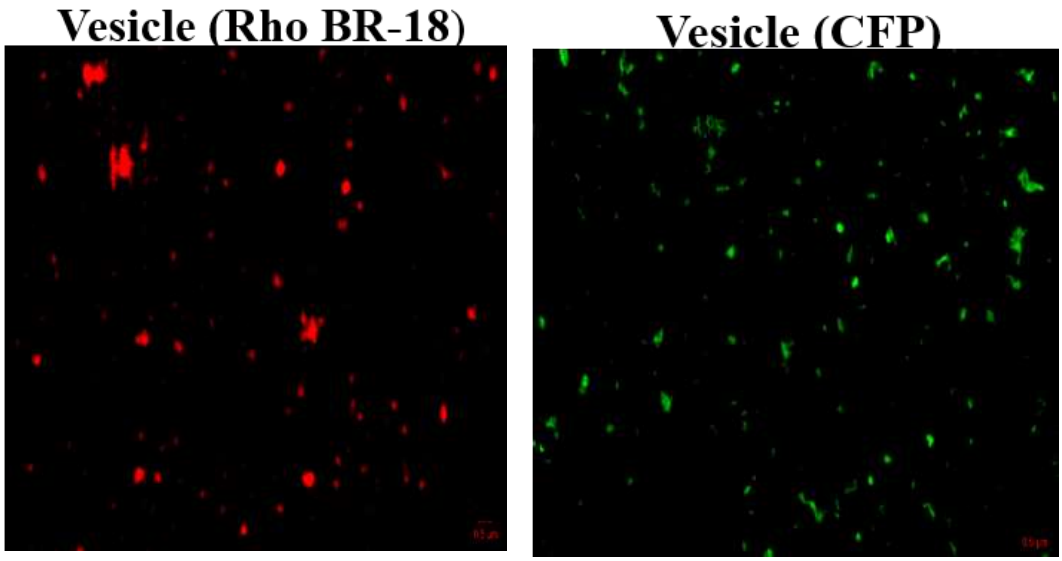


Fig 2.5 Super resolution microscopy (SR-SIM) Image of purified MVs. 2 μg of MVs were stained with rhodamine BR-18 or 2μg purified MVs from CFP expressing *Y. pseudotuberculosis* ATCC29833 were imaged under super resolution microscope (Scale Bar 500nm)

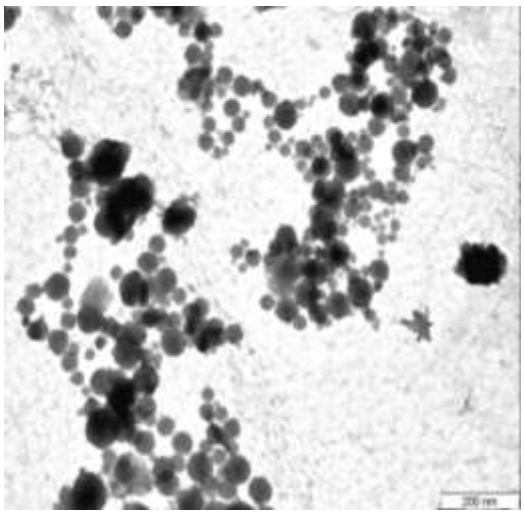


Fig 2.6 TEM images of purified MVs from *Y. pseudotuberculosis* ATCC29833. It clearly shows heterogeneous population of MVs the size is varied greatly between approximately 20 nm to 400 nm in diameter (scale bar 200nm)

2.4.3 Packaging of Chaperonin into the MVs

Table 1 lists the most common protein as the 60-kDa chaperonin. The strikingly high-level presence of this protein within the MVs suggested that it was being packaged. To study this further, a recombinant His-tagged chaperonin in the pET31b vector was expressed within *Y. pseudotuberculosis* ATCC29833. Using antibodies specific for this epitope, the presence of the chaperonin within both the whole cell and the MV fractions was assayed (**Fig 2.5**). No cross reactivity was seen when the control samples, *i.e.*, wild-type whole cells or MVs, were probed using this antibody. However, immunoblots of the recombinant samples show that the quantity of chaperonin within the MVs was much greater than that of the corresponding whole cells. Semi-quantification of the chaperonin present in the MVs was performed using purified recombinant protein as a standard (**Fig 2.6**) and suggests that the MVs may contain as much as ten-times more chaperonin per microgram of protein than the whole cells. It should be considered that the complementation of chaperonin with plasmid will lead to increase in the gene copy number. So we determined the copy number of the pET31b(+) plasmid in *Y. pseudotuberculosis* ATCC 29833 and YPIII (+) strain by qPCR method using CNFy as an internal control(single copy in chromosome). Result confirms the strains grown at 30°C copy number was found to be 60 and 35 of peT31B+ plasmid ATCC29833 and YPIII(+) at 37°C copy number was 35 and 41 in ATCC29833 and YPIII (+) respectively **(Fig 2. 20 and Table 2.6)**

The high level presence of the chaperonin protein within the vesicles was also seen in other bacterial pathogens, including *Franscella* [88], giving credence to our findings but also implying that this chaperonin may be being packaged into MVs. When the proteins within MVs from *E. coli* were identified, we once more found the chaperonin from this bacterium to be one of the most concentrated proteins. Although not definitive, the continued high-level presence of the chaperonin within MVs of different bacterial species suggests that MVs may be a common means employed by virulent bacteria to disseminate this virulence factor to the host cells. As such, the mechanism used by bacteria to package the chaperonin into MVs is clearly an area that needs to be studied further. This apparent packaging of the chaperonin into the MVs was not growth stage dependent as MVs purified from cultures grown for 4, 8, 12 hours contained.

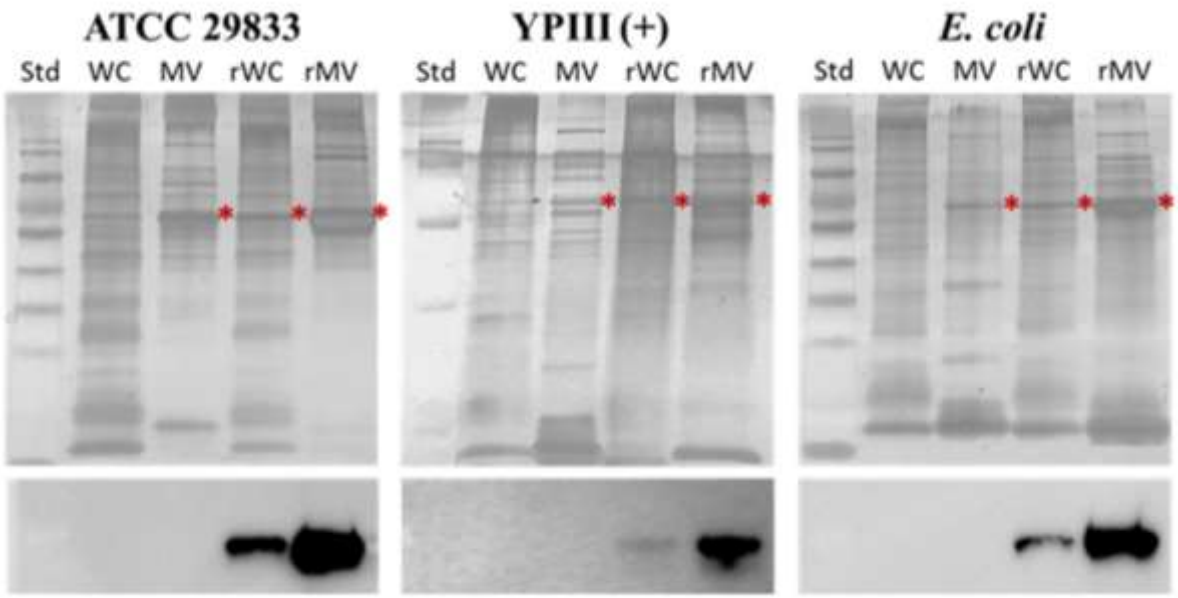


Fig 2.7 Comparative analysis of the proteins present within the whole cells and those in the purified MVs. Std is the protein marker, wild type bacteria (WC) membrane vesicle from wild-type bacteria (MV) recombinant bacteria expressing Chaperonin (rWC) MVs from recombinant bacteria(rMV). For SDS-PAGE gel analysis sample were treated with SDS- Sample buffer then quantified by Bradford assay 15ug of protein from each sample loaded. Subsequently performed Silver staining and western blotting Using anti-His HRP conjugate.

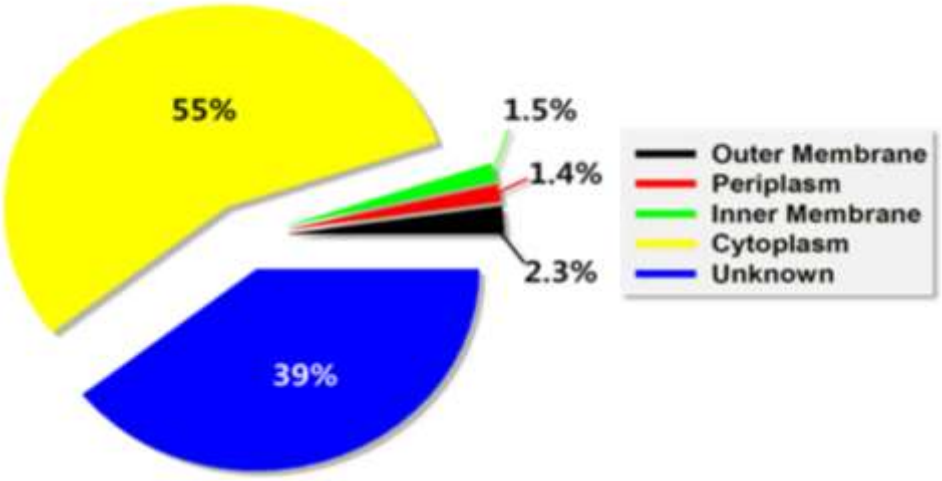


Fig 2.8 Pie chart showing subcellular localization of protein associated with *Y. pseudotuberculosis* ATCC 29833 MVs. Subcellular localization was determined by using PSORTb database.

2.4.4 *Y. pseudotuberculosis* MVs Invade Human Cells and Cause Multinucleation

As a human pathogen, *Y. pseudotuberculosis* is probably best known for its ability to invade into human cells via a β-actin mediated endocytotic mechanism involving the bacterial INV or AilA proteins (The ability of this strain to invade NuLi-1 airway epithelial cells illustrated in **Fig2.10**, where CFP-expressing whole bacterial cells are evident within the epithelial cells shortly after their addition. Tests with the MVs purified from *Y. pseudotuberculosis ATCC* cultures also retain the ability to invade as shown by the CFP-fluorescent MVs visibly present within the human epithelial cells (**Fig 2.10**).Further confirmation MVs were stained with Rhodamine BR-18, stained MVs were allowed to Invade NuLi-1 **(Fig 2.9)** cell and MCF 10a Cells**(Fig 2.10)** result confirmed the MVs were internalized in NuLi-1 and MCF-10a Cells Time lapse Image of YPIII+ vesicle treated cells shows the vesicle can be retained inside the cells up to 24 hours **(Fig 2.17)**

One may easily surmise from the data in **Table 2.1** and **Figs 2.8** that the MVs are being used to introduce the associated virulence factors into the human cells when they invade. This was confirmed in Fig 2B as the recombinant chaperonin could be detected within the infected human cells via western blotting (**Fig 2.13**). For each sample, 20 µg of total protein was separated by SDS-PAGE.

Not only are virulence factors being transported by MVs but they also appear to be active as clear dose-dependent phenotypic responses were elicited from the exposed NuLi-1 epithelial cells (**Fig 2.14 and 2.15**). **Fig 2.15** shows epithelial cells exposed for 3 hours to 10 µg/ml of purified *Y. pseudotuberculosis* ATCC MVs displaying extensive β-actin rearrangement. Similar results were also obtained when MVs from *Y. pseudotuberculosis* YPIII (+) were tested. Likewise, in both tests, an exposure to the MVs also led to the formation of giant cells, a phenotype associated multinucleated cells

Moreover, the viabilities of the epithelial cells at 48 hours decreased in a dose-dependent manner as greater quantities of purified MVs were added, with a 5 and 10 µg/ml addition of *Y. pseudotuberculosis* ATCC MVs leading to a 50% and 70% loss in viability, respectively (**Fig2.14**). Parallel tests performed using MVs purified from *Y. pseudotuberculosis* YPIII (+) found slightly weaker but similar dose-dependent losses (**Fig 2.14**). To further assert that the toxicity resulted from the MVs and not some other soluble protein present within the preparation, the purified MVs were removed by 100kD filtration. When the filtrates from both *Y. pseudotuberculosis* strains were tested, no adverse effect on the viability was observed (**Fig 2.16**). Moreover, pre-treatment of the purified MVs with either heat or proteinase K before their addition significantly or completely alleviated their toxicity, respectively (**Fig 2.14**).

2.4.5 *Y. pseudotuberculosis* MVs Transport CNFy into Human Cells

Although the MVs from both *Y. pseudotuberculosis* strains led to similar losses in viability and widespread β-actin rearrangement and fiber formation within the epithelial cells, one clear difference between the MVs from these two related strains was seen in their ability to cause multinucleation (**Fig 2.18**). Whereas the degree of multinucleation was dose-dependent for both preparations, these Figs show that the *Y. pseudotuberculosis* YPIII (+) MVs are far more potent and lead to a significantly greater degree of multinucleation than those from *Y. pseudotuberculosis* ATCC 29833.

Multinucleation of the human cells strongly hinted at the presence of the cytotoxic necrotizing factor (CNFy), a known virulence factor leading to this phenotype It is also known that the gene encoding for CNFy is full-length in *Y. pseudotuberculosis* YPIII (+) (3.1 kb) but is truncated in *Y. pseudotuberculosis* ATCC (1.8 kb), which helps to explain the different phenotypes seen with the exposed epithelial cells[70] To determine if CNFy is being transported with the MVs, and that the full-length protein can account for the differences seen for these two pathogens, the full-length CNFy gene (3.1 kb) was cloned and expressed within *Y. pseudotuberculosis* ATCC 29833. As shown in (**Fig 2.18)**, expression of the full-length CNFy gene within this host led to significantly more multinucleation and at a level that is analogous to that seen with *Y. pseudotuberculosis* YPIII (+) MVs. That was, confirming that the CNFy protein is responsible for this phenotype and that it is very likely associated with MVs. To confirmed the presence of CNFy in the MVs initially western blot analysis was not succeeded due to CNFy further the MVs sample were subjected to ELISA (**Fig 2.15**) and confirmed CNFy present in the MVs

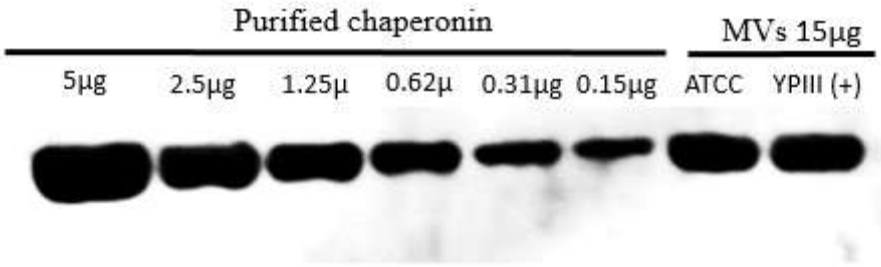


Fig 2.9 Semi-quantitative analysis of the chaperonin present in the MVs. Semi-quantification of the chaperonin present in the MVs was performed using purified recombinant protein as a standard.

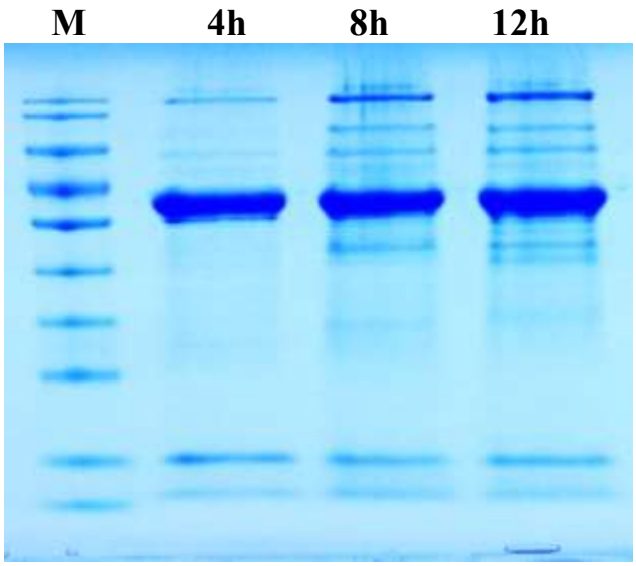


Fig 2.10 Packaging of chaperonin in MVs at the different time point of *Y. pseudotuberculosis* ATCC29833 growth. MVs purified at different time points 4, 8, 12 and 15µg of MVs subjected to SDS-PAGE page followed coomassie blue staining.

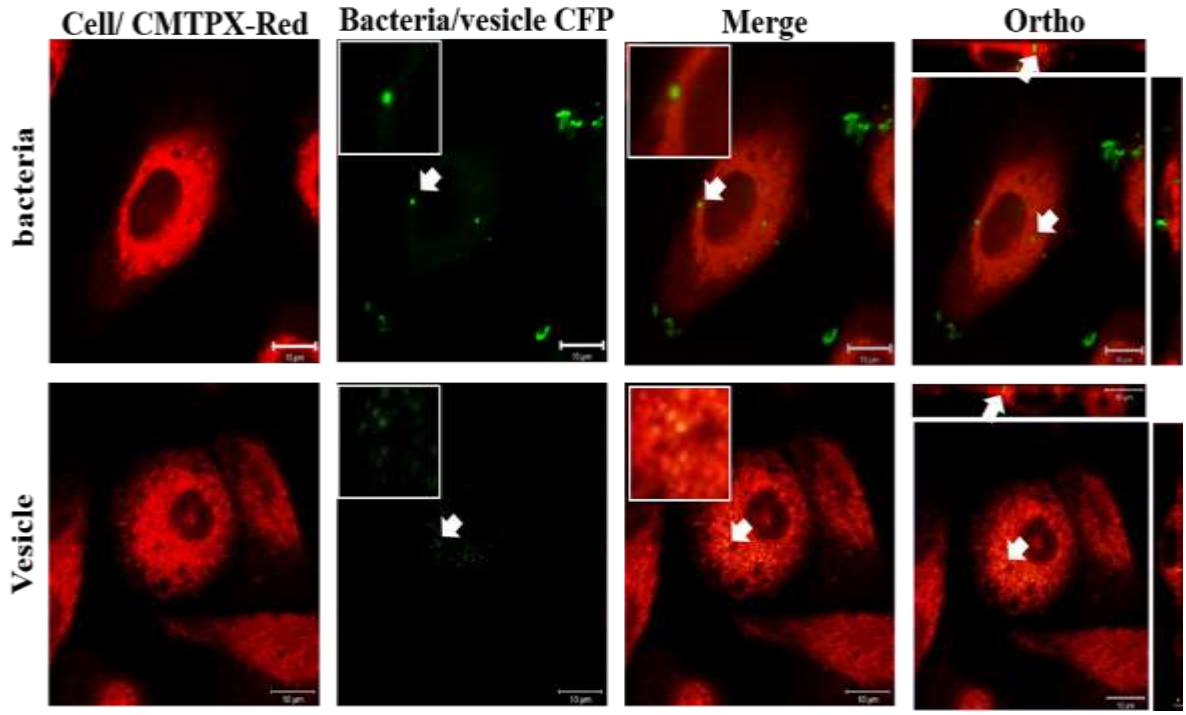


Fig 2.11 Invasion of *Yersinia pseudotuberculosis* ATCC 29833 MVs into NuLi-1 cells Image shows localization of *Y. pseudotuberculosis* live bacteria, and MVs invaded into airways epithelial cell, 85% confluent epithelial cell were stained with cell tracker CMTPX- Red stain then co-cultured with CFP expressing *Y. pseudotuberculosis* approximately 200 bacteria/cell (bacteria). 10ug purified MVs from CFP expressing bacteria were treated to the cells (MVs). After 2 hours non invaded bacteria and MVs were remove by washing with DPBS and media were replaced with media supplemented with antibiotics time and imaged using laser conafocal microscope white arrow indicate location of MVs.(Scale bar 10 μm)

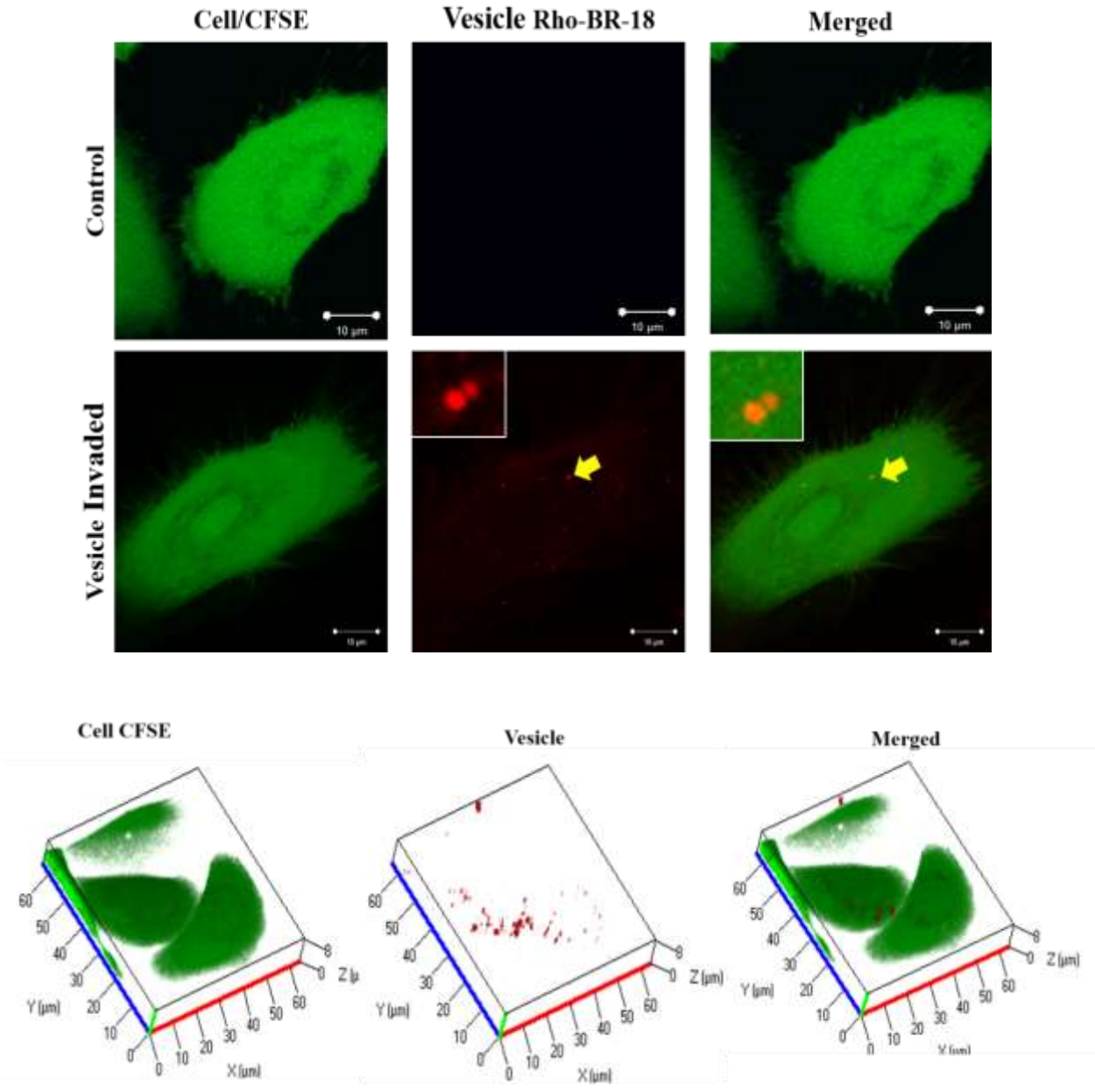


Fig 2.12 Rhodamine BR-18 labeled MVs invaded into with NuLi-1 cells. Image shows localization of *Y. pseudotuberculosis* ATCC 29833 invaded MVs inside the airways epithelial cell, 85% confluent epithelial cell were stained with cell CFSE cell proliferation stain then treated with 10µg of purified MVs for 2 hours. After incubation, non-invaded MVs were removed by washing with PBS 3 times and imaged using laser confocal microscope yellow arrow, indicate location of MVs (Scale bar 10 µm)

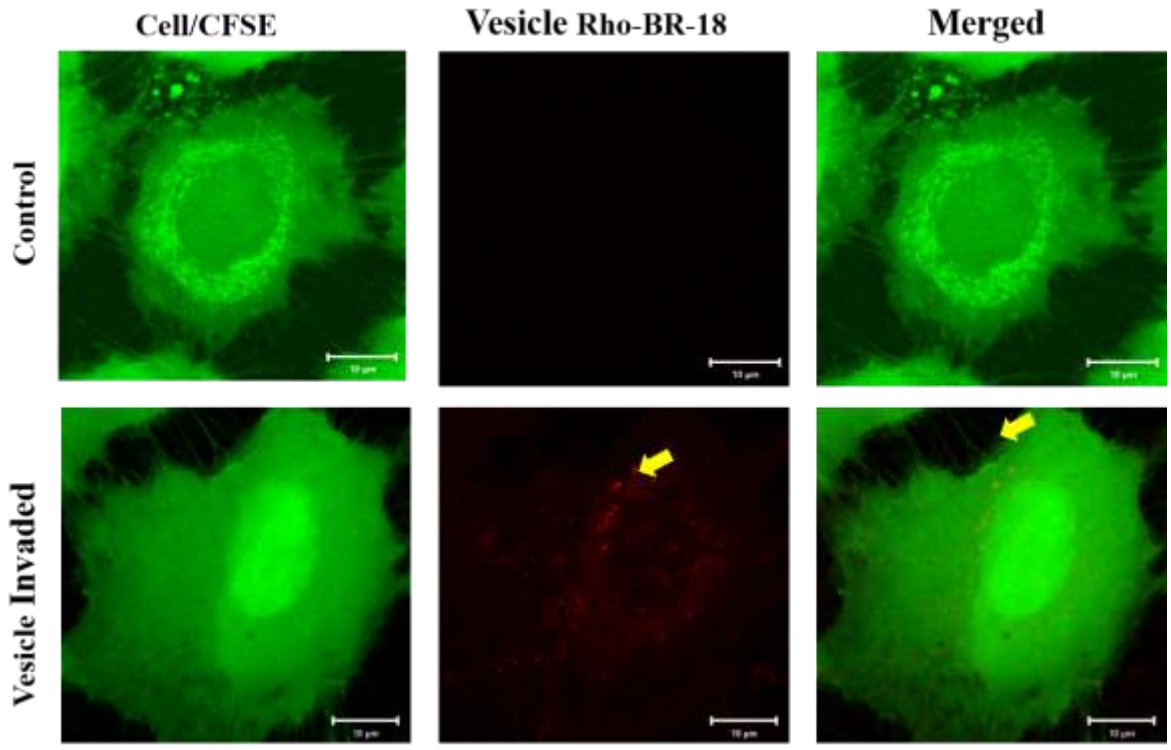


Fig 2.13 Rhodamine BR-18 labeled MVs invaded into with MCF-10a cells. Image shows localization of *Y. pseudotuberculosis* ATCC29833 invaded MVs inside the airways epithelial cell, 85% confluent epithelial cell were stained with cell CFSE cell proliferation stain then treated with 10µg of purified MVs for 2 hours. After incubation non invaded MVs were removed by washing with PBS 3 times and imaged using laser confocal microscope yellow arrow, indicate the location of MVs (Scale bar 10 µm)

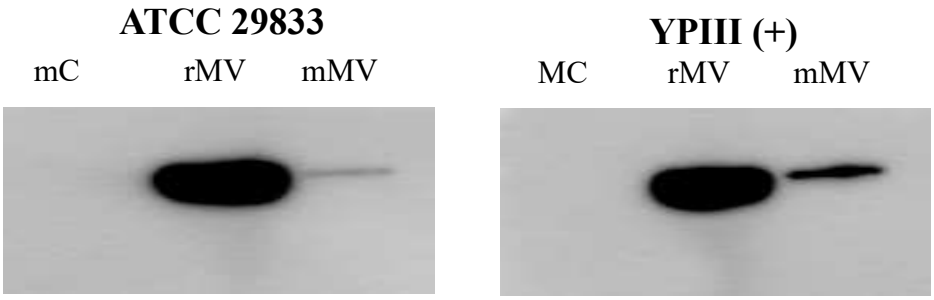


Fig 2.14 Recombinant 60kDa chaperonin detected within the mammalian cells. NuLi-1 cells were treated with 20 ug of Purified MVs which from *Y. pseudotuberculosis* ATCC29833-Chap and YPIII(+)-Chap. MVs were allowed to invade for 2 hours then treated cells were washed with DPBS to remove unbound MVs then cells were trpsinzed the trypsinzed cells were boiled with SDS-PAGE sample buffer then 20 µg of total protein was separated by SDS-PAGE subsequently analysed by western blotting using Anti -His HRP conjugate. mC is the media control, rMV- MVs with recombinant His-tagged 60kDa Chaperonin control (20 ug of MVs) invaded MVs into NuLi-1 cells.

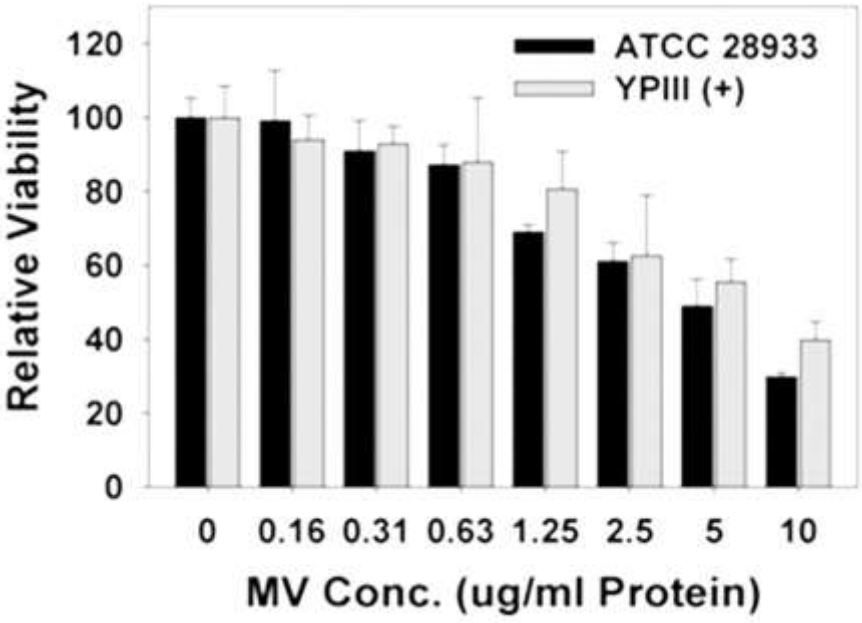


Fig 2.15 Viabilities of the NuLi-1 after 48 hours of MVs treatment. The viability of NuLi1 cell decreased in a dose-dependent manner as greater quantities of purified MVs were added, with a 5 and 10 µg/ml addition of *Y. pseudotuberculosis* ATCC MVs leading to a 50% and 70% loss of viability.

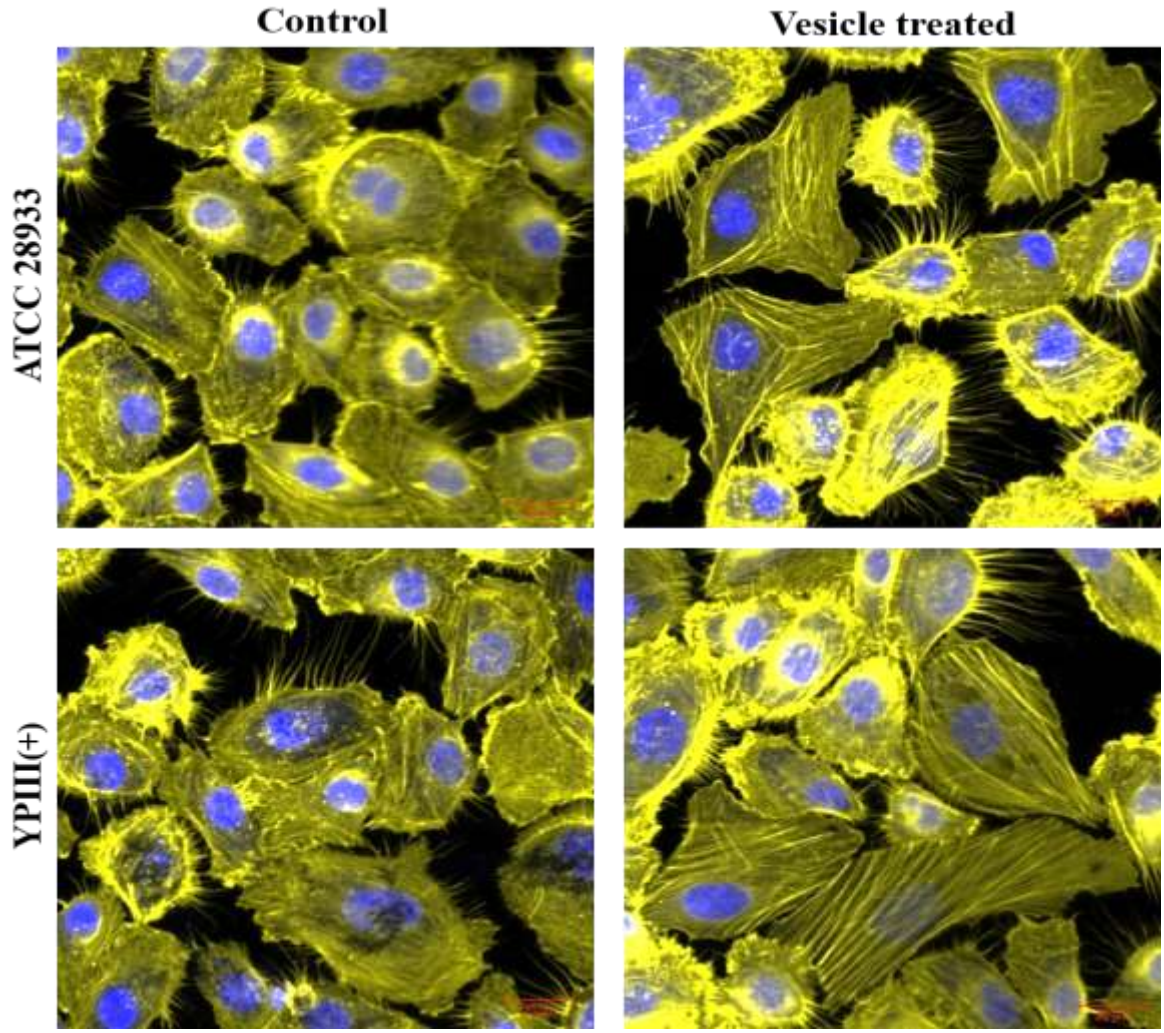


Fig 2.16 Purified *Y. pseudotuberculosis* ATCC29833 and YPIII (+) MVs displaying extensive β-actin rearrangement after 3hour of treatment with 10 µg/ml MVs. 10 µg/ml Purified MVs from the both strain to the NuLi-1 cells for 3 hours. After treatment cells were washed and fixed with 3.7 % of paraformaldehyde and washed with DPBS then stained Rhodamine-phalloidin, counterstained with DAPI then image were taken by confocal microscopy at 100X magnification (scale bar 20µm)

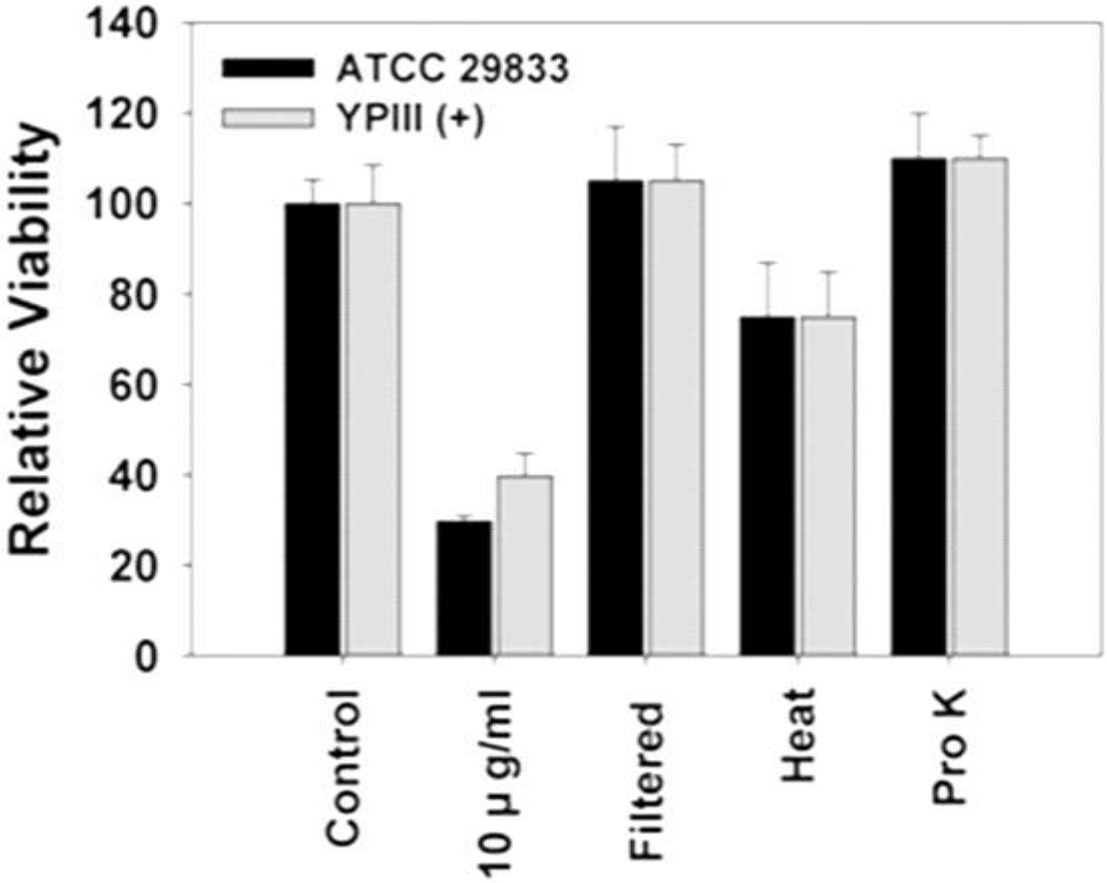
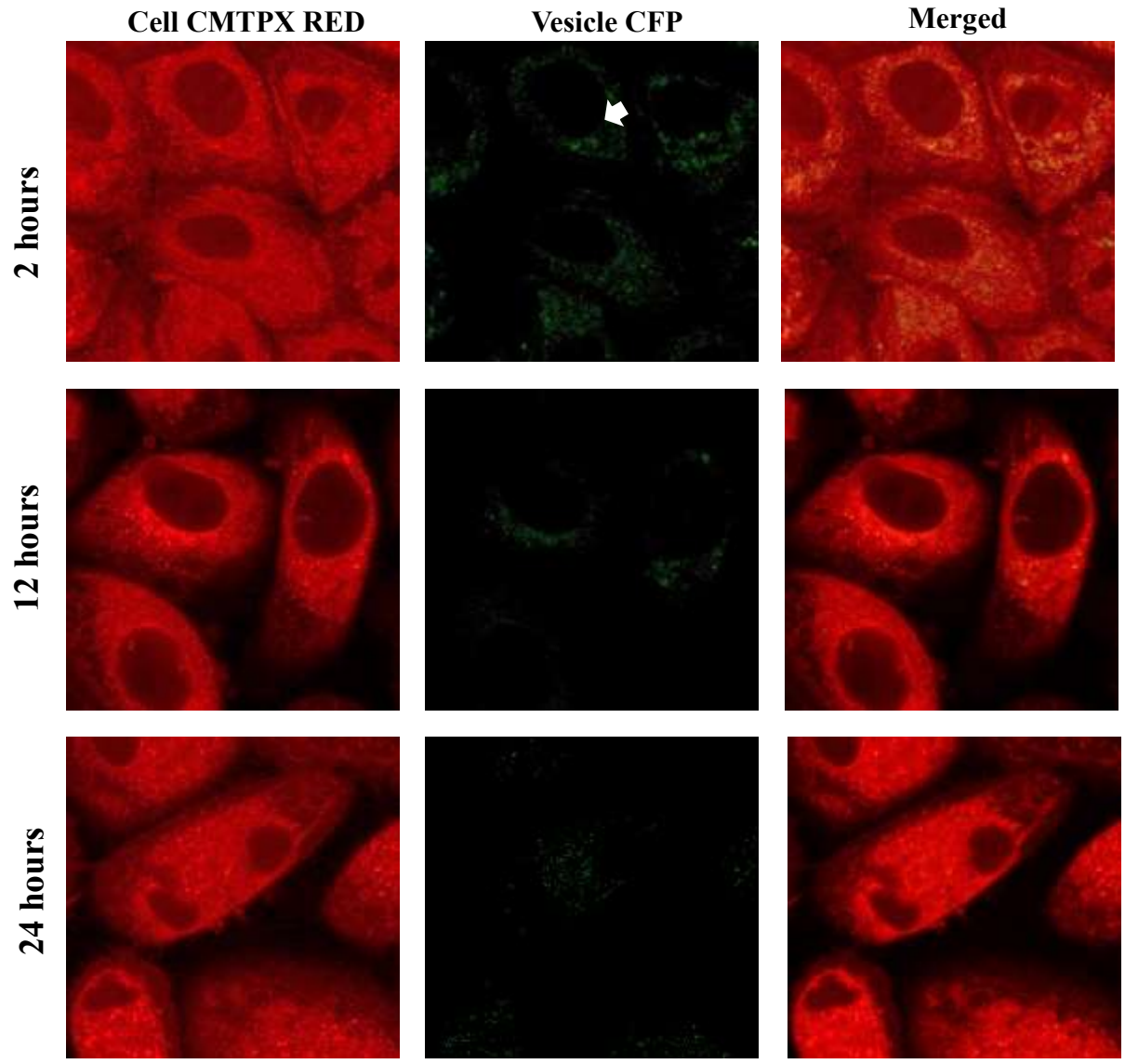


Fig 2.17 Cytotoxic effect of Different fraction of *Y. pseudotuberculosis* MVs on NuLi-1 cells. Toxicity of MVs on NuLi-1 cell. To confirm the toxicity resulted from the MVs and not some other soluble protein purified MVs Subjected to Different treatments. Media No MVs (Control) Vesicle (10μg/ml) 100kDa filtration flow thought (Filtered), heat treated MVs (Heat) Vesicle treated with Proteinase K (Pro K).



2.18. Time lapse image shows localization of *Y. pseudotuberculosis* YPIII(+) MVs invaded into Nuli-1. 85% confluent epithelial cell were stained with cell tracker CMTPX- Red 20 µg purified MVs from CFP expressing *Y. pseudotuberculosis* YPIII (+) were treated with the cells. After 2 hours unbound MVs were removed by washing with DPBS then cells are replenished with fresh media supplemented with antibiotics. Time lapse image were taken at different time intervals (Scale bar 10µm)

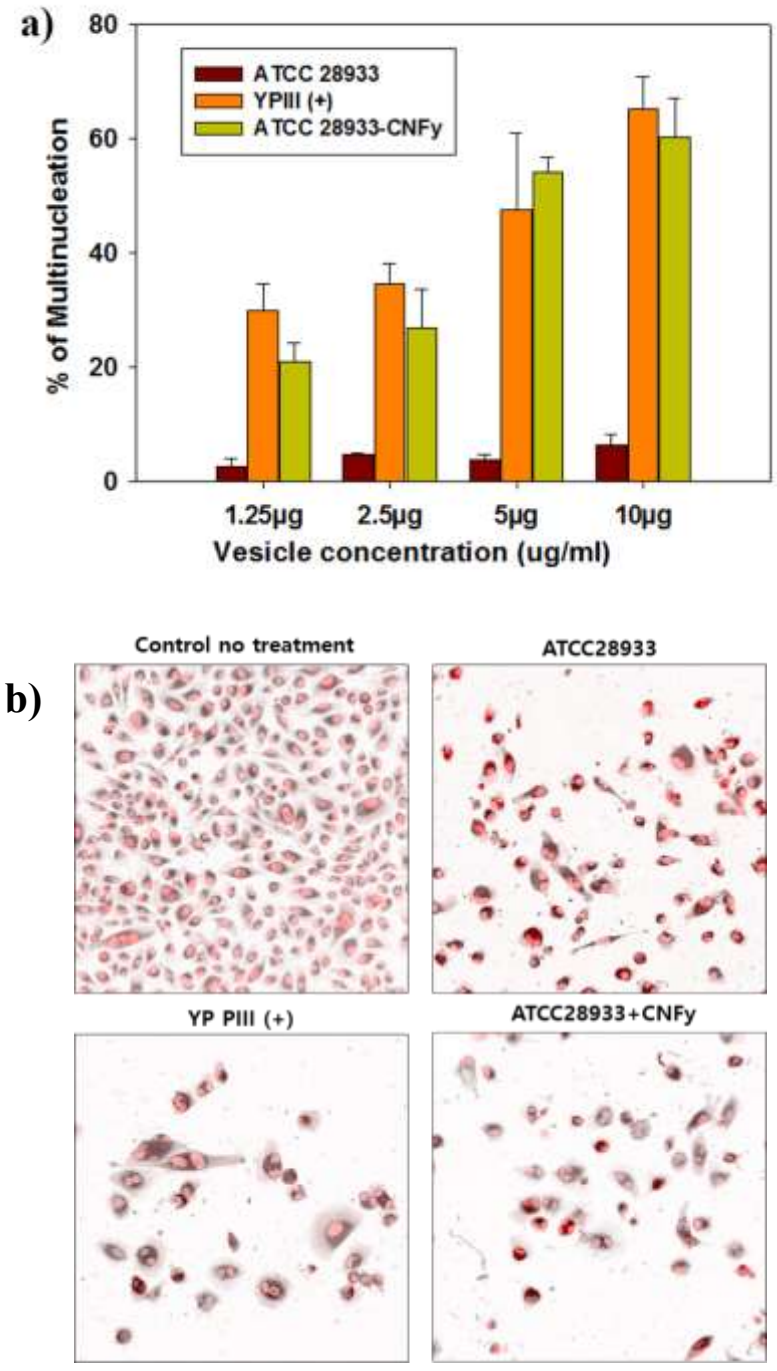


Fig 2.19 Determination of multinucleated cells after treatment of MVs a) Percentage of multinucleated cells were determined by imaging followed by counting. The used various concentration of MVs 1.5ug to 10 µg. Vesicle from ATCC29833 YPIII+ and ATCC29833 with recombinant CNFy. **b)** Representative image of cells treated with 10µg of MVs.

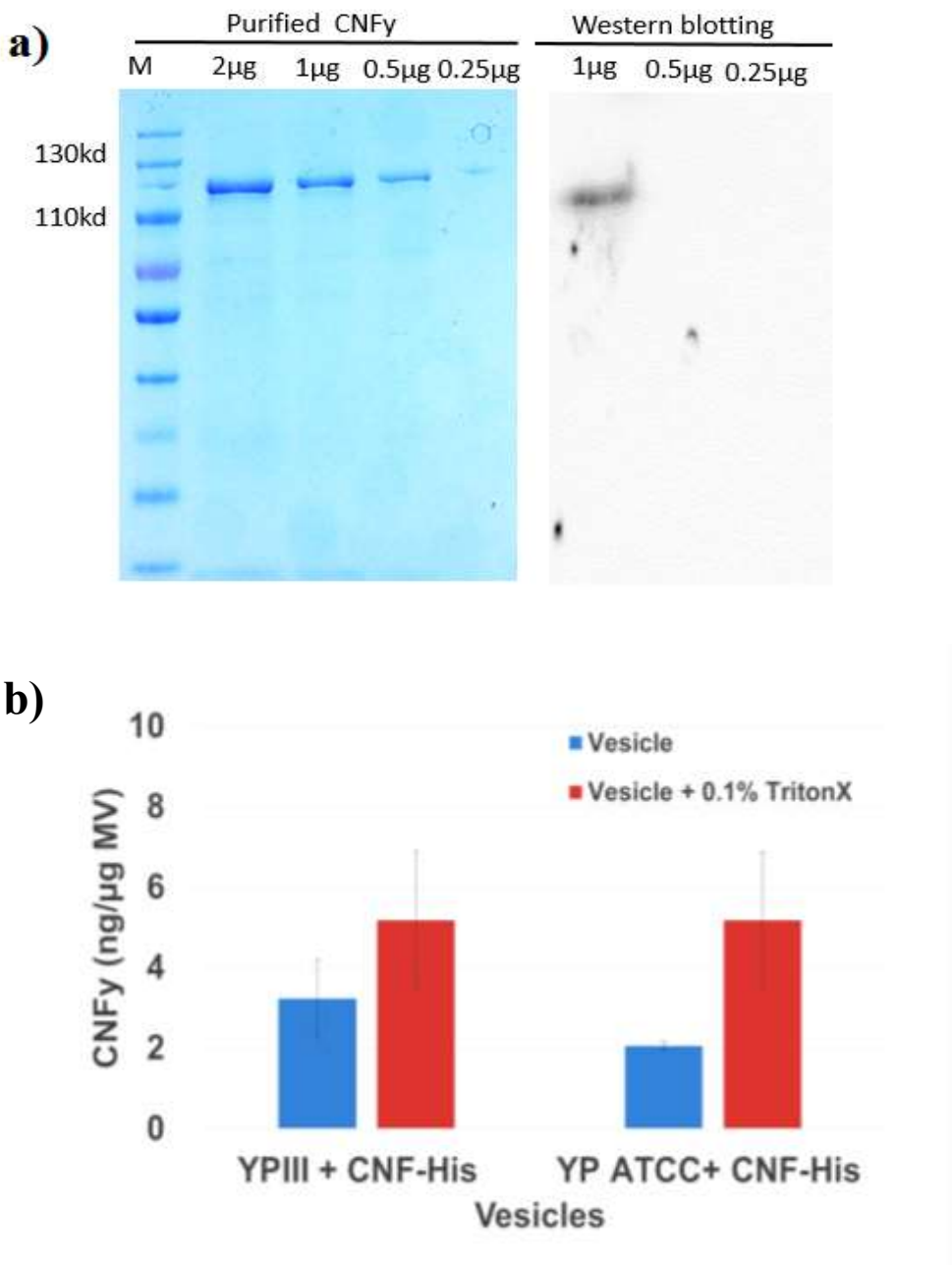


Fig 2.20 Quantification of CNFy associated with MVs by ELISA using His-Probe HRP conjugate
a) SDS –PAGE gel and western blot analysis of purified CNFy purified CNFy were loaded at different concentration. **b)** 10μg of purified membrane vehicle were treated with 0.1% of triton X and non-treated were coted on 96 well plate. Know concentration of purified CNFy-His expressed in YPIII (+) was used to plot the stranded curve.

Table 2.1 Pathogenesis-related protein associated with *Y. pseudotuberculosis* ATCC29833 MVs

	Protein	**Uniprot ID**	**MW**	**Peptides**	**Localization**
1	60 kDa chaperonin	CH60_YERP3	57 kDa	4941.167	Cytoplasmic
2	Urease subunit alpha	URE1_YERP3	61 kDa	757.2433	Cytoplasmic
3	Urease subunit beta	B1JR70_YERPY	17 kDa	166.7433	Cytoplasmic
4	Peptidase B	PEPB_YERPB	47 kDa	224.19	Cytoplasmic
5	Attachment invasion locus protein	AIL_YERPS	20 kDa	67.337	Outermembrane
6	Peptidase M16	B2JZ59_YERPB	108 kDa	39.81133	Cytoplasmic
7	Outer membrane protein,	A7FI37_YERP3	23 kDa	34.664	Outermembrane
8	Protease Do	B1JK15_YERPY	50 kDa	33.20533	Unknown
9	Protein tyrosine/serine phosphatase	B1JP79_YERPY	31 kDa	30.96733	Cytoplasmic/membrane
10	Outer membrane protein A	A7FJS9_YERP3	39 kDa	30.604	Outermembrane
11	Porin Gram-negative type	B1JS88_YERPY	41 kDa	24.68667	Outermembrane
12	Lipoprotein	A7FFJ7_YERP3	29 kDa	21.01333	Outermembrane
13	Lipoprotein	B2K715_YERPB	23 kDa	20.41167	Outermembrane
14	Peptidase M48	B2K0S3_YERPB	27 kDa	20.07467	Cyto/ membrane
15	Peptidase M24	B2K0Q9_YERPB	49 kDa	18.62567	Cytoplasmic/membrane
16	Major outer membrane lipoprotein	LPP_YERPS	8 kDa	15.04	Outermembrane
17	Phospholipase	B1JIM9_YERPY	52 kDa	12.0846	Outermembrane
18	General Bacterial Porin	Q66CG8_YERPS	40 kDa	11.74393	Outermembrane
19	Peptidase M22	B1JLJ4_YERPY	25 kDa	11.02333	Cytoplasmic
20	Peptidase U32	B1JLW5_YERPY	37 kDa	10.7027	Cytoplasmic
21	Oligopeptidase A	A7FP13_YERP3	77 kDa	9.608067	periplasmic
22	17 kDa surface antigen	B1JJ74_YERPY	15 kDa	9.589833	Unknown
23	Aminopeptidase N	B2JYR6_YERPB	98 kDa	9.507533	Cytoplasmic
24	Putative lipoprotein	Q66AB7_YERPS	29 kDa	5.871033	Outermembrane
25	Oligopeptidase B	B1JP33_YERPY	79 kDa	6.0087	Cytoplasmic
26	Peptidase M24	B1JME6_YERPY	45 kDa	4.9846	Cytoplasmic
27	Putative chaperone protein	B1JSE3_YERPY	49 kDa	4.4743	periplasmic
28	Putative lipoprotein	B1JPB8_YERPY	19 kDa	5.118867	Outermembrane
29	Phospholipase A	Q7B171_YERPU	34 kDa	3.941933	Outermembrane
30	Hemolysin	YERP3-DECOY	-	1.297867	Outermembrane

Table 2.2 Outer membrane protein associated with *Y. pseudotuberculosis* ATCC29833 MVs

S.No	Protein	Uniprot ID	MW/Kda	Peptide No
1	Attachment invasion locus protein	AIL_YERPS	20 kDa	67.337
2	Outer membrane protein, OmpW	A7FI37_YERP3	23 kDa	34.664
3	Outer membrane protein A	A7FJS9_YERP3	39 kDa	30.604
4	Nitrilase/cyanide hydratase and apolipoprotein	B1JKG9_YERPY	32 kDa	30.40133
5	Porin Gram-negative type	B1JS88_YERPY	41 kDa	24.68667
6	Lipoprotein	A7FFJ7_YERP3	29 kDa	21.01333
7	Lipoprotein	B2K715_YERPB	23 kDa	20.41167
8	Outer membrane protein assembly factor	B1JS08_YERPY	42 kDa	16.71167
9	Major outer membrane lipoprotein	LPP_YERPS	8 kDa	15.04
10	Phospholipase D/Transphosphatidylase	B1JIM9_YERPY	52 kDa	12.0846
11	General Bacterial Porin (GBP) family protein	Q66CG8_YERPS	40 kDa	11.74393
12	VacJ family lipoprotein	B1JGF8_YERPY	28 kDa	8.775433
13	Type III effector Hrp-dependent outers	B1JS75_YERPY	46 kDa	8.449733
14	Outer membrane protein assembly factor BamA	BAMA_YERP3	88 kDa	6.949067
15	Membrane protein	B1JGF9_YERPY	45 kDa	6.6753
16	Outer membrane protein assembly factor BamC	B2K994_YERPB	38 kDa	5.8878
17	Putative lipoprotein	Q66AB7_YERPS	29 kDa	5.871033
18	Maltoporin 1	LAMB1_YERP3	47 kDa	5.306767
19	Phospholipase A	Q7B171_YERPU	34 kDa	3.941933
20	Hemolysin	YERP3-DECOY	---	1.297867

Table 2.3: Top 20 cytoplasmic proteins associated with *Y.pseudotuberculosis* ATCC 29833 MVs

S. No	Protein	Uniprot ID	*MW/kDa*	Peptide No
1	60 kDa chaperonin	CH60_YERP3	57 kDa	4941.167
2	DNA-directed RNA polymerase subunit beta rpoC	RPOC_YERP3	155 kDa	1516.633
3	DNA-directed RNA polymerase subunit beta rpoB	RPOB_YERP3	150 kDa	1225.427
4	Pyruvate dehydrogenase E1 component	A7FM42_YERP3	100 kDa	930.2833
5	Urease subunit alpha ureC	URE1_YERP3	61 kDa	757.2433
6	Pyruvate dehydrogenase,	Q66EH9_YERPS	55 kDa	682.23
7	Iron-containing alcohol dehydrogenase	B1JKU8_YERPY	97 kDa	645.0133
8	DNA-directed RNA polymerase subunit alpha rpoA	RPOA_YERP3	37 kDa	498.52
9	Glutamine synthetase	B1JR07_YERPY	52 kDa	437.7567
10	Malate dehydrogenase	B1JSJ4_YERPY	82 kDa	425.0067
11	Aspartate ammonia-lyase	B1JMR4_YERPY	53 kDa	354.41
12	Ribose-phosphate pyrophosphokinase	B1JM90_YERPY	34 kDa	348.0467
13	FAD dependent oxidoreductase	B1JHY2_YERPY	57 kDa	246.3333
14	Peptidase B	PEPB_YERPB	47 kDa	224.19
15	Glyceraldehyde-3-phosphate dehydrogenase, type I	B1JLG3_YERPY	36 kDa	213.9867
16	Phenylalanine-tRNA ligase beta subunit	SYFB_YERPS	87 kDa	205.2767
17	Ribonuclease R	B1JMN1_YERPY	95 kDa	200.96
18	Citrate synthase OS=Yersinia pseudotuberculosis	B1JG64_YERPY	48 kDa	191.2867
19	Formate acetyltransferase	B1JRE6_YERPY	85 kDa	181.3467
20	Urease subunit beta ureB	B1JR70_YERPY	17 kDa	166.7433

Table 2.4: Periplasmic proteins associated with *Y. pseudotuberculosis* outer MVs

S.No	Protein	Uniprot ID	MW/kDa	Peptide No
1	Protein TolB	TOLB_YERP3	46 kDa	15.706
2	L-asparaginase, type II	B1JRE3_YERPY	36 kDa	14.22033
3	Aldose 1-epimerase	B1JLG4_YERPY	32 kDa	10.03797
4	Glycerophosphodiester phosphodiesterase	B1JP88_YERPY	27 kDa	7.509633
5	Oligopeptidase B	B1JP33_YERPY	79 kDa	6.0087
6	Thiol:disulfide interchange protein	B1JR14_YERPY	23 kDa	5.442867
7	Murein tripeptide ABC transporter	A7FHP5_YERP3	60 kDa	5.256533
8	Ecotin	ECOT_YERP3	19 kDa	4.522933
9	Putative chaperone protein	B1JSE3_YERPY	49 kDa	4.4743
10	Oligopeptide/dipeptide ABC transporter, ATPase subunit	B1JJR9_YERPY	38 kDa	4.232433
11	Oligopeptide/dipeptide ABC transporter, ATPase subunit	B1JKU2_YERPY	37 kDa	3.293933
12	Formate dehydrogenase-O, major subunit	Q663U7_YERPS	113 kDa	3.260433
13	Putative periplasmic beta-glucosidase	A7FE47_YERP3	80 kDa	1.945867

Table 2.5 Bacterial plasmid and primers used in this study

Strain or plasmid	Description/genotype	Reference
Y. pseudotuberculosis ATCC 29833	Wild type strain	-
Y. pseudotuberculosis YPIII +	Wild type strain	-
Y. pseudotuberculosis ATCC 29833 CFP	*Y. pseudotuberculosis* ATCC 29833 with pAmcyan	This study
Y. pseudotuberculosis YPIII CFP	*Y. pseudotuberculosis* YPIII(+) with pAmcyan	This study
Y. pseudotuberculosis ATCC 29833-Chap	*Y. pseudotuberculosis* ATCC 29833 With pET31b-Chap-His	This study
Y. pseudotuberculosis YPIII(+)- Chap	*Y. pseudotuberculosis* YPIII (+) Chap-His	This study
Y. pseudotuberculosis29833 CNF	*Y. pseudotuberculosis* ATCC 29833 With pET31b-CNFy-His	This study
Y. pseudotuberculosis YPIII(+) CNFy	*Y. pseudotuberculosis* YPIII (+) CNFy-His	This study
E .coli DH5-Alpha		
E .coli BW NlpI chap	*E coli BW NLpI* pET31b-Chap-His	This study
E.coli BW NlpI CNF	*E coli BW NlpI* pET31b-CNFy-His	This study
Plasmids		
pAmCyan	Plasmid expressing pAmCyan	
Pet 31b +	Expression Vector	
Pet 31 B+ Chap	Plasmid expressing Chaperonin	This study
Pet 31 b+ CNFy	Plasmid expressing CNFy	This study
Primers		
Chap F	5’ AATCTAGATGTGAAAGTCGGCGACGTCG 3’	This study
Chap R	5’ATTCTCGAGCATCATGCCGCCCATGC 3’	This study
CNF F	5’TATTCTAGATTTTACATGTAATAATAAATAGCAG3’	This study
CNF R	5’ATACTCGAGAAAGTCTTTTTGTAAAACATTAAAC3’	This study

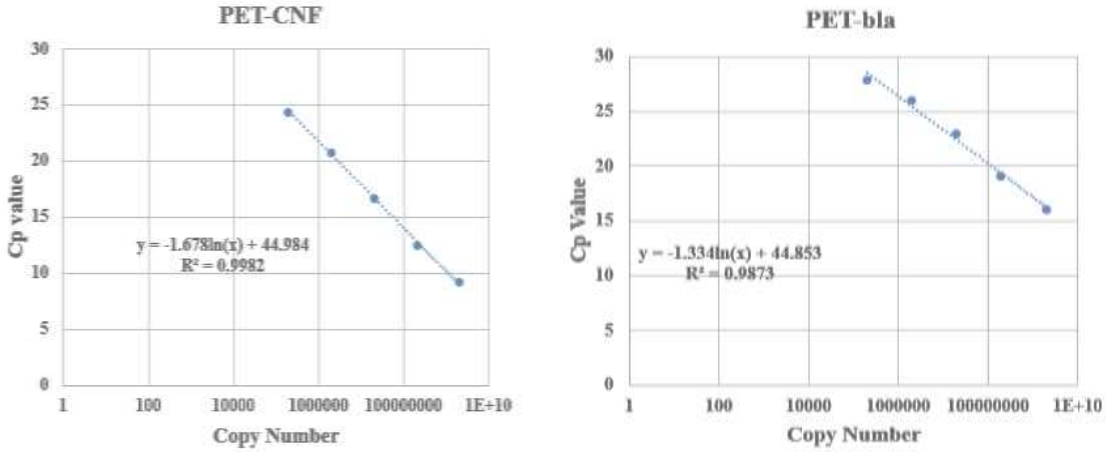


Fig 2.21 based on Cp value obtained from qRT PCR. Standard curve drawn for cytotoxic necrotizing factor CNFy and beta-lactamase gene

Table 2.6 The copy number of the pET31b+ plasmid in both *Y. pseudotuberculosis* strain at different growth condition

Strains Condition	**Gene copy/ µl**		**Plasmid copy**
30°C	**CNFy gene**	**bla gene**	
YPIII(+)	2950447	102176230	35.003661(+4.2)
ATCC29833	2857973	171628443	60.279306(15.37)
37°C			
YPIII(+)	2821241	106736726	37.846198 (1.55)
ATCC29833	3085280	129846008	41.640521 (11.16)

2.5 Conclusion

Yersinia pseudotuberculosis produces MVs. Transmission electron microscope and super resolution microscopy analysis revealed 24 hours grown *Y. pseudotuberculosis* was associated with MVs. Further proteomic analysis by LC-MS/MS confirmed the 887 vesicular proteins with high confidence by three separate runs. The result revealed that MVs contains various virulence factors. Based on subcellular localization predictions, MVs are composed of an outer membrane, periplasmic and cytoplasmic proteins. Further studies with human airway epithelial cells, purified MVs induced morphological changes in human airway epithelial cells, and MVs were shown to be cytotoxic as they are internalized by the human airway epithelial cell. Further confirmation, *Y. pseudotuberculosis* MVs deliver cytotoxic necrotizing factor (CNFy) into the host (Fig 2.17). In this study we demonstrated novel secretion system which *Yersinia* utilizes to deliver CNFy and other virulence factors into the host system.

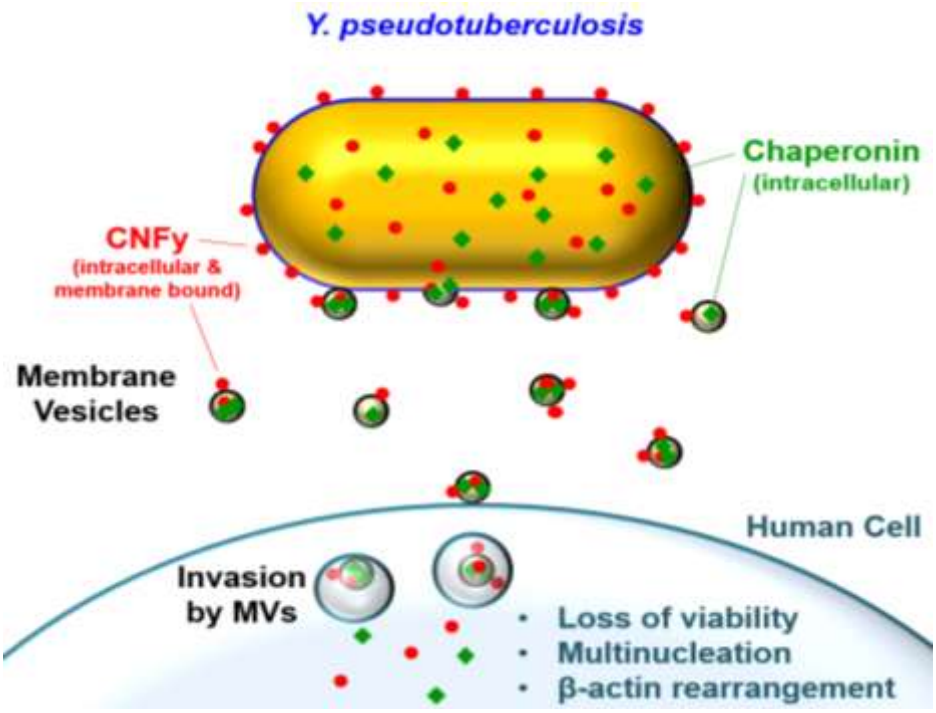


Fig 2.22 Possible mechanism which *Y. pseudotuberculosis* membrane vesicle deliver virulence factors of CNFy and 60kDa Chaperonin into Human cells

Chapter 3

Yersinia pseudotuberculosis Membrane Vesicles Are Cytotoxic and Induce TNF-Alfa in Mouse Leukemic Monocyte/Macrophage Raw 264.7

3.1 Summary

Yersinia pseudotuberculosis membrane vesicles (MVs) adverse effect on human airway epithelial cells (NuLi-1) was confirmed in the previous study. The present study focused on the response of immune cells murine macrophage RAW264.7 to MVs. Experimental infection of *Y. pseudotuberculosis* ATCC 29833 and YPIII(+) revealed both associated with macrophages after 2 hours of coinfection with MOI of 300. Furthermore, murine macrophages were treated with purified MVs from CFP expressing *Y. pseudotuberculosis* ATCC 29833 and *Y. pseudotuberculosis* YPIII (+) strains. The result suggested that MVs are also associated with murine macrophages after 2 hours of treatment. Furthermore, the treatment with MVs leads to actin rearrangement after 3 hours of incubation, and there was a significant loss in viability after 48 hours of exposure to the MVs. Toxicity was in a dose-dependent manner. 12 µg/ml of the MVs showed a significant loss in viability, and it was about 53.4 % and 42.1%. 200 µg/ml showed 93.84% and 84.6% loss in viability from ATCC29833 and YPIII(+), respectively. The result revealed giant cell multinucleated phenotype observed after 48 hours of exposure to MVs. Further study showed that macrophages have induced a high level of TNFα response to purified vesicles 5209 pg/ml, and 5214 pg/ml TNFα were detected in both ATCC and YPIII(+) vesicle-treated cells, and non-treated was 190.6 pg/ml after 24 hours of exposure. Apoptosis assay confirmed ATCC 29833 and *Y. pseudotuberculosis* YPIII(+) MVs treated macrophages that underwent 1.38% and 2.5% apoptosis. 8.3% and 9.1% of cells died after 24 hours from cells treated with ATCC29833 and YPIII MVs. The result suggests that the apoptosis is not a significant event in cytotoxicity. Further time-lapse image treatment with *Y. pseudotuberculosis* YPIII(+) MVs showed the vesicles are associated with the macrophages 24 hours of post infection of membrane vesicles.

3.2 Introduction

Macrophages, together with other professional phagocytic cells including neutrophils and monocytes, play a crucial role in the host-defense system through recognition and elimination of invading pathogenic bacteria[89]. In the initial stage of bacterial infection, immune cells will come in contact with the pathogen[90]. The phagocytosis of the bacteria, the survival or death of macrophages, is crucial for the host's frontline immune defense[91]. Macrophages either promote their instantaneous survival or derail the system-level immune response.[92] Gram-negative bacteria secrete virulence factors, and the secreted virulence factors are essential for the survival of the bacteria, or they damage the host system[9]. The section of virulence factor is a complicated process because of the complex structure of bacterial

envelope[93]. Pathogens have various strategies to secrete virulence factors. Virulence factors enable to gain access to the extracellular environment, typically the tissues or bloodstream of the host organisms. There are various secretion systems: Type I to Type VI. These secretion systems are known to deliver virulence factors that are soluble in nature, but the membrane vesicles deliver MVs that are very diverse complexes of pathogen-associated molecular patterns (PAMPs),[71] such as lipopolysaccharides, membrane protein, virulence factors, and cell envelope lipids.[94] The composition of MVs is different from that of other species, but the lipopolysaccharide structure remains relatively constant within a bacterial species.[95] The presence of PAMPs, virulence factors, and other outer membrane components associated with MVs helps to be recognized by the immune system.[96]

The host is immune to MVs mainly by the generation of antibodies to the MVs and their components. Some studies reported antibody production and protection in response to outer membrane vesicles from *Neisseria meningitidis*[97] and *Vibrio cholerae*[98]. In contrast, there are relatively few studies characterizing how vesicles trigger the innate inflammatory response[99]. There are a few studies that reported inflammatory response from epithelial cells,[100], but the macrophage is limited. In the present study, we focused on characterizing macrophage responses to the outer membrane vesicles. In some reports was shown MVs play an important role in activating innate immune responses[101]. Given their small size and a high proportion of PAMPs, it is reasonable to expect that vesicles could easily infiltrate into infected tissues and stimulate widespread inflammation[95]. In addition to TLR ligands, vesicles also contain a variety of protein virulence factors that may specialize vesicles for specific functions or types of host cell damage.

Yersinia pseudotuberculosis is a food borne pathogen, and it is transmitted via the fecal-oral route. It infects the gut-associated lymphoid tissue.[102] It is well known that *Yersinia pseudotuberculosis* delivers YOPs protein via Type III section system to modulate the host's immune system.[69] Cytotoxic necrotizing factor enhances the delivery of Yops into murine macrophages that are responsible for initiating inflammatory defenses against colonizing bacteria.[95] The previous study demonstrated *Yersinia pseudotuberculosis* membrane vesicle is known to contain various virulence proteins and purified MVs showed a dose-dependent cytotoxicity and led to actin rearrangement and multinucleation in human airway epithelial (HAE) cell line, NuLi-1. In this study, we focused on characterizing the response of macrophage to MVs.*Yersinia pseudotuberculosis* MVs significantly influenced both the macrophage morphology and the TNF-alfa responses. Based on this study, we concluded macrophages showed a very distinct response to the membrane vesicles. The study suggested that the response is due to the effect of heterogeneous factors associated with vesicle protein by macrophages.

3.3 Materials and Methods

3.3.1 *Bacteria* growth and culture condition

Y. pseudotuberculosis ATCC 29833 from obtained from KACC and *Y. pseudotuberculosis* YPIII (+) NR-4380 with virulence plasmid(+) was obtained from BEI Resource and was kept in frozen glycerol stocks at -80°C. Upon need, bacteria were streaked on a nutrient LB (Difco, USA) agar plate and incubated at 30°C overnight. From this plate, a single colony was inoculated into 5 ml of LB broth and incubated overnight at 30°C with agitation (200 rpm). The fluorescent bacteria were developed by transferring both stains with a pAmCyan plasmid (Promega, USA) and selected in ampicillin 100μg/ml plate. The recombinant fluorescent bacteria were grown on Luria-Bertani (LB) plate supplemented with ampicillin.

3.3.2 Mouse leukemic monocyte/macrophage RAW 264.7 cell culture

The mouse macrophage cell line RAW 264.7 ATCC TIB-71 was obtained from Professor Sang Sun Yoon of Yonsei University, Korea. The cells were cultured in DMEM F12 medium (Gibco; Life Technologies, NY, USA), supplemented with 10% fetal bovine serum (FBS; ATCC, USA) and with hormone 100 mg/ml (InvivoGen, USA) at 37°C with 5% CO_2 incubator.

3.3.3 MVs isolation and purification

To purify MVs, *Y. pseudotuberculosis* ATCC 29833-CFP and *Y- pseudotuberculosis* YPIII (+)-CFP followed previously reported method with slight modification. Single colonies were inoculated into 5 ml of LB broth for 24 hours. Then the overnight grown culture was diluted 1:100 in 500 ml of LB ampicillin media in a 2-liter conical flask and incubated at 30°C for 24 hours. After incubation, the cells were removed by pelleting (4000 RPM, 20 min) in 500 ml of centrifuge bottle, and then supernatants were collected and filtered twice with a 0.22-micron filtration unit (startup and strip; Millipore, USA). After filtration, the MVs were concentrated using a 100-kDa tangential filtration concentration unit (Amicon Ultra-15; Millipore, USA) to approximately 5 ml. Concentrated MVs pelleted by centrifuging at 40,000 RPM for 2 hours were suspended in phosphate-buffered saline (PBS). The concentration of MVs was quantified based on the protein content using the Bradford colorimetric assay method.

3.3.4 Invasion of *Yersinia pseudotuberculosis* and purified MVs

For visualization of invasion, a monolayer of macrophage was prepared on 8 well-chambered cover glass (Nunc® Lab-Tek® II; Thermo Scientific, USA) by seeding $2.5X10^4$ macrophage cells/well and incubated in a CO_2 incubator at 37ºC for 24 hours with 5% CO_2. Before MVs treatment, RAW cells were stained with cell tracker CMTPX Red (Life Technologies, USA) by replacing media with 0.5 PBS containing stain of a final concentration of 5µg/ml and incubated at 37 ºC in a CO2 incubator for 10 min. After incubation, cells were washed twice with PBS and were added to 0.5 ml of DMEM F12 media. Stained cells were incubated for 1 hour at 37ºC in a CO_2 incubator with 5% CO_2. For invasion assay, overnight-grown bacteria were diluted in DMEMF12 media up to the final MOI of 300 and purified MVs from CFP expressing *Yersinia* were diluted to 10µg/ml in DMEM F12 media. Initially, well was replaced with 0.5 ml of DMEMF12 with MVs incubated for 2 hours at 37 ºC in a CO_2 incubator with 5% CO_2. After incubation had unbound, MVs were replaced with 0.5 ml DMEM F12 media. Then cells were imaged at 100x magnification using LSM 700 laser scanning confocal microscope (Carl Zeiss, Germany) operated by ZEN 2009 software.

3.3.5 Cytotoxicity assay

For determining the viability of the cultured host cells, a monolayer of macrophage cells was prepared on a 96 well plate by seeding $1X10^4$ RAW cells/well and incubated in a CO_2 incubator at 37ºC for 24 hours. Then it was replaced with 100 µl media. With different MVs, the cells were kept until 48. With MVs treatment and the MTT assay done, approximately 40 µl of the MTT reagent were added to each well with control and treatment wells and incubated at 37ºC for 2 hours in the dark. After incubation, the whole liquid was decanted and soaked to remove any remaining liquid in any well by tapping on paper towel thoroughly. About 400 µl DMSO were added to each well and incubated with shaking at room temperature for 15 minutes. Development of visualized color and OD was measured at 550 nm. The viability of cultured RAW cells was proportional to the OD value.

3.3.6 Apoptosis assay

For determining the viability of the cultured host cells, a monolayer of RAW was prepared in a 35 mm culture dish by seeding $1X10^5$ RAW 264.7 cells /well and incubated in a CO_2 incubator at 37ºC with 5% CO_2 for 24 hours. After incubation, the cells were washed twice with DPBS then infected with 1 ml purified MVs in DMEM F12 in 10 µg/ml media without MVs as a control. Initially, well was replaced with 0.5 ml of DMEMF12 with MVs and without MVs as control and incubated for 24 hours at 37ºC. After 24 hours, the cells were trypsinized and washed twice with Dulbecco's phosphate-buffered saline (DPBS). $1X10^5$ suspension cells in 100 µl Annexing V binding buffer were added to 5 µl of Annexing

V and PI according to the manufacturer's recommendations. Incubate the tubes in the dark for 15 minutes at the room. The stained cells were analyzed by FACS (BD FACS Calibur, USA), and the data were analyzed using FlowJo Software.

3.3.7. Immunostaining of F-actin

For F-actin staining, a monolayer of RAW cells were prepared on 8 well-chambered cover glass by seeding $2.5X10^4$/well and incubated in a CO_2 incubator at 37ºC for 24 hours. To study the influence of the MVs on actin cytoskeleton rearrangements, the 24-hour culture was serum starved for 20 hours, and then cells were washed with DPBS and incubated with purified MVs 500 µl (5µg) or control media for 3 hours. Subsequently, cells were fixed with 3.7 % paraformaldehyde (in DPBS) for 20 min at room temperature. After fixation, the cells were washed with DPBS and permeabilized with 0.2% Triton-X (Sigma-Aldrich, USA) in PBS for 5 min. The actin cytoskeleton was stained with 0.5 µg/ml of rhodamine phalloidin (Invitrogen, USA) for 1 hour at room temperature. The cells were washed with DPBS, and the nuclei were stained with DAPI 1µg/ml (Life Technologies, USA) in DPBS for 5 min at room temperature. The cells were visualized using a laser confocal microscope LSM 700 operated by Zeiss, Germany.

3.3.8 Observation of multinucleation

To determine the multinucleation, RAW cells (25000 cells /well) were seeded or grown on 96 well plate for 24 hours with 5% $C0_2$, then incubated with different concentration of MVs for 48 hours. After incubation, the cells were fixed with 3.7 % paraformaldehyde for 10 min at room temperature. Fixed cells were washed with DPBS and stained with CMTPX red and DAPI. Then the cells were visualized under a microscope at 10X magnification, and taken image.

3.3.9 Quantification of TNF-alfa

RAW cells were grown on a 12 well plate (SPL, Korea) $5X10^4$ cells/well for 24 hours, and then replaced media with vesicle with 10 µg/ml of MVs (5 µg/well) media without vesicle used as a control Treated cells were incubated in a CO_2 incubator at 37ºC for 24 hours. After 24 hours, cell culture media were collected for ELISA. TNF-α concentration was quantified using ELISA kits from R&D Systems, USA, and was performed according to the manufacturer's instruction.

Result and Discussion

3.4.1 *Y. pseudotuberculosis* MVs associated with macrophages

Most of the *Y. pseudotuberculosis* strains will replicate in the macrophages, except the *Y. pseudotuberculosis YPIII* (+) strain, which is defective in nature and unable to replicate in murine macrophages. Replication is independent of type III secretion systems.[103] We began our study by confirming that *Y. pseudotuberculosis* invades murine macrophages. Initially, macrophages were infected with CFP expressing both ATCC29833 and YPIII (+) (MOI of 300) and 5 µg (10 µg/ml). After 2 hours of infection, unattached bacteria and MVs were removed by washing with DPBS consequently treated with 50µg/ml gentamycin then fixed with 3.7% paraformaldehyde and visualized under a confocal microscope. The microscopic image clearly indicates that the *Y. pseudotuberculosis* live bacteria and MVs are associated with macrophages after 2 hours of infection, which stained red macrophages associated with CFP expressing live bacteria were invaded and some of the bacteria found to be associated with the surface of the RAW cells **(Fig 3.1).** The MVs associated with the RAW cells were clearly visible **(Fig 3.2).** The localization of CFP packaged MVs demonstrated that the MVs carry cytoplasmic contents. Future treated with only 20 µg/ml YPIII vesicle. The vesicles are associated with the macrophage, and initially, after 2 hours, the vesicles were observed in endocytic vesicles, and images were taken after 12 hours. When the endocytic vesicles become brighter, it suggests that the vesicles are ruptured, or the pH of the endocytosis vesicle was increased. After 24 hours, the vesicles seem to be dispersed and the florescence **(Fig 3.3).** Unfortunately, CFP is very sensitive to acidic pH, losing ~50% of its fluorescence at pH 6.5. The initial stage of coinfection with the vesicle appears to be very less florescent. It is believed that the endocytic vesicle will change during endocytosis to reduce the lapse image of murine macrophage. It will become one of the brightest and most widely utilized of the FPs.

3.4.2 *Y. pseudotuberculosis* MVs is cytotoxic to murine macrophage

In the previous study on airway epithelial NuLi-1 cells, the MVs showed significant cytotoxicity. To determine the possible effect of MVs on macrophage, we tested the toxicity of MVs using MTT assay. The cytotoxicity of MVs at a different concentration determined by treating RAW cells with 200 µg/ml to 3 µg/ml. The viability of cells was after 48 hours of MVs treatment. The result suggested that purified MVs are cytotoxic to murine macrophage **(Fig 3.3).** The viability was decreased in a dose-dependent manner. 25 µg/ml of the MVs showed 50% loss of viability. The highest concentration of MVs 200 µg was up to 90% loss in viability compared with the 84% to 95% survival of 3 µg/ml MVs treatment **(Fig 3.3).** Treatment with ATCC 29833 MVs was significantly more toxic than with YP III (+) vesicles. The result was similar to NuLi-1 cell, which suggests that both MVs packaging will be different. The LPS

of the bacteria induces cytotoxicity, and MVs also contain LPS. The previous report suggests that macrophages were more sensitive to MVs compared with LPS. [104]

3.4.3 Apoptosis is not major event in cytotoxicity

Membrane vesicle from the pathogenic bacteria showed to induce apoptosis. LPS also one of the inducers of apoptosis. In our study, we try to understand the mechanism behind the cytotoxicity so we analyzed MVs treated macrophage for apoptosis and necrosis. MVs treated RAW cells were analyzing cells by FACS using apoptosis marker Annexin V, the cells were grown on 6-well plate seeded $5X10^5$ cells/well and incubated for 24 hours in a CO_2 incubator with or without membrane vesicle. Then cells were trypsinized and stained with Annexin V and propidium iodide, a dead stain. The result suggests that around 1.4% and 2.5% ATCC29833 and YPIII(+) MVs treated cells are showing apoptosis signature **(Fig 3.4)** subsequently stained with propidium iodide around 8.1% and 9.3% ATCC29833 and YPIII(+) treated cells represented as dead. It suggests that cytotoxicity is not allied with the apoptosis. Suggests that there may be other factors responsible for the cell death. Based on the previous report on *in-vivo* studies, the *Y. pseudotuberculosis* infection leads to inflammation and necrosis, but to YopJ, intracellular expression is needed for the survival of bacteria that induce apoptosis.[105]

3.4.4 *Y. pseudotuberculosis* MVs treatment leads to morphological changes in murine macrophages RAW 264.7

Further tests macrophage cells were incubated with purified MVs from ATCC29833,YPIII(+) and, respectively. After 4 hours, treated cells were fixed and stained with rhodamine-phalloidin and analyzed by fluorescence microscopy. As expected, YPIII (+) MVs induced the actin rearrangement and led to, filopodia observed on the cell surface **(Fig 3.4)**. The morphology of cells and MVs-induced actin rearrangements observed with YPIII(+) MVs and ATCC 29833, respectively **(Fig 3.5),** were different. As shown in, incubation of RAW cells with 29833 for 4 hours had no effect on the morphology of RAW cells and morphology resembled the control cell. Further evaluation, treated cells were incubated for 48 hours and stained with CMTPX red, and DAPI showed the giant cells phenotype observed, and all cells are searched **(Fig 3.6)** the result suggested that the *Y. pseudotuberculosis* MVs deliver CNFy, which leads to actin rearrangement and filopodia. Further observation revealed that raw cells were treated with purified MVs from both YPIII(+) and ATCC 29833 after 48 hours of MVs showed multinucleation **(Fig 3.6).** This effect is mainly due to cytotoxic necrotizing factors.

3.4.5 *Y. pseudotuberculosis* MVs induces secretion of tumor necrosis factor-alpha (TNF-α) in murine macrophage

Murine macrophages were treated with *Y. pseudotuberculosis* MVs 10 µg/ml from both ATCC29833 and YPIII (+), media without MVs as a control. After 24 hours, culture media were collected and quantified the production of TNF-alfa. The result confirms MVs from both induce a high level of proinflammatory cytokine TNF-alfa 5209 pg/ml, and 5214 pg/ml from ATCC29833 and YPIII (+) respectively. The tumor necrosis factor alpha cytokine is mainly associated with type I cellular immunity. TNF-alfa requires the protection of the host cell from *Yersinia* infection.[106] TNF-α counteracts the apoptotic pathway. The activation of MAP kinases and NF-κB, two key components of the pro-inflammatory pathway, leads to the secretion of TNF-α.[105]

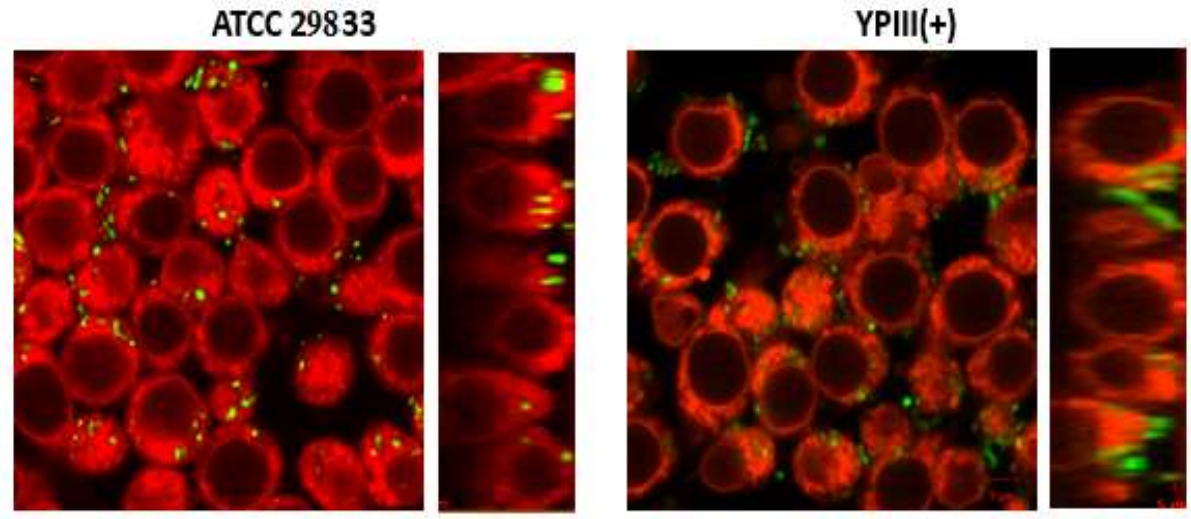


Fig 3.1 Laser confocal microscope image of *Y. pseudotuberculosis* ATCC 29833 and YPIII (+) associated with murine macrophage RAW 264.7 cells. Overnight-grown *Yersinia* strain infected to RAW cells at the MOI of 300. After two hours of infection, un-infected bacteria were removed by washing with DPBS and subsequently treated with 50μg/ml of gentamycin for 15 min and fixed with 3.7% paraformaldehyde. For visualization, macrophages were stained with CellTracker CMTPX red (scale bar 2 μm).

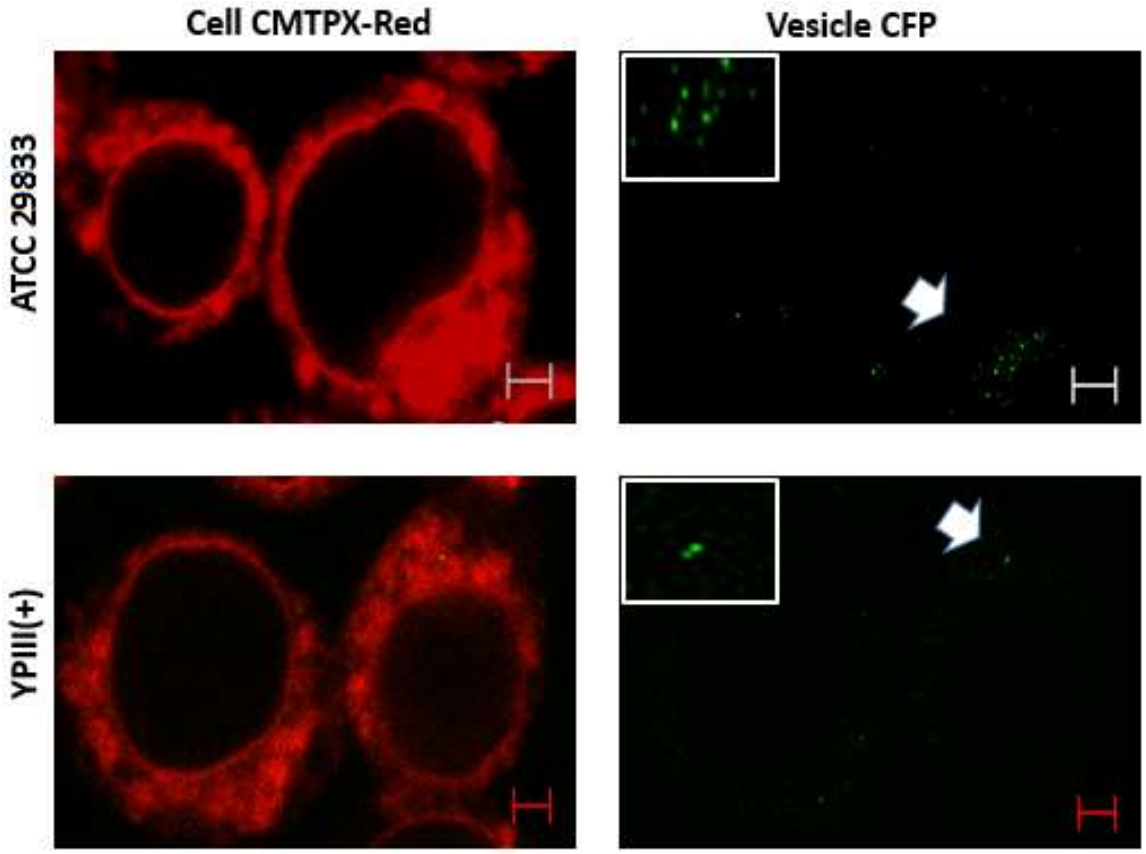


Fig 3.2 Confocal image of *Y. pseudotuberculosis* MVs associated with murine macrophage. 80% confluent macrophages were prepared on 8 chambered cover glass. Before infection macrophage was stained with CMPTX red. For visualization of MVs 5µg (10 µg/ml) purified MVs from CFP expressing ATCC 29833 and YPIII (+) were treated with macrophage cells for 2 hours. After two hours, unattached MVs were removed by washing with PBS and subsequently treated with 50µg/ml of Gentamycin for 15 min and fixed with 3.7% paraformaldehyde then visualized under a microscope (scale bar 2µm).

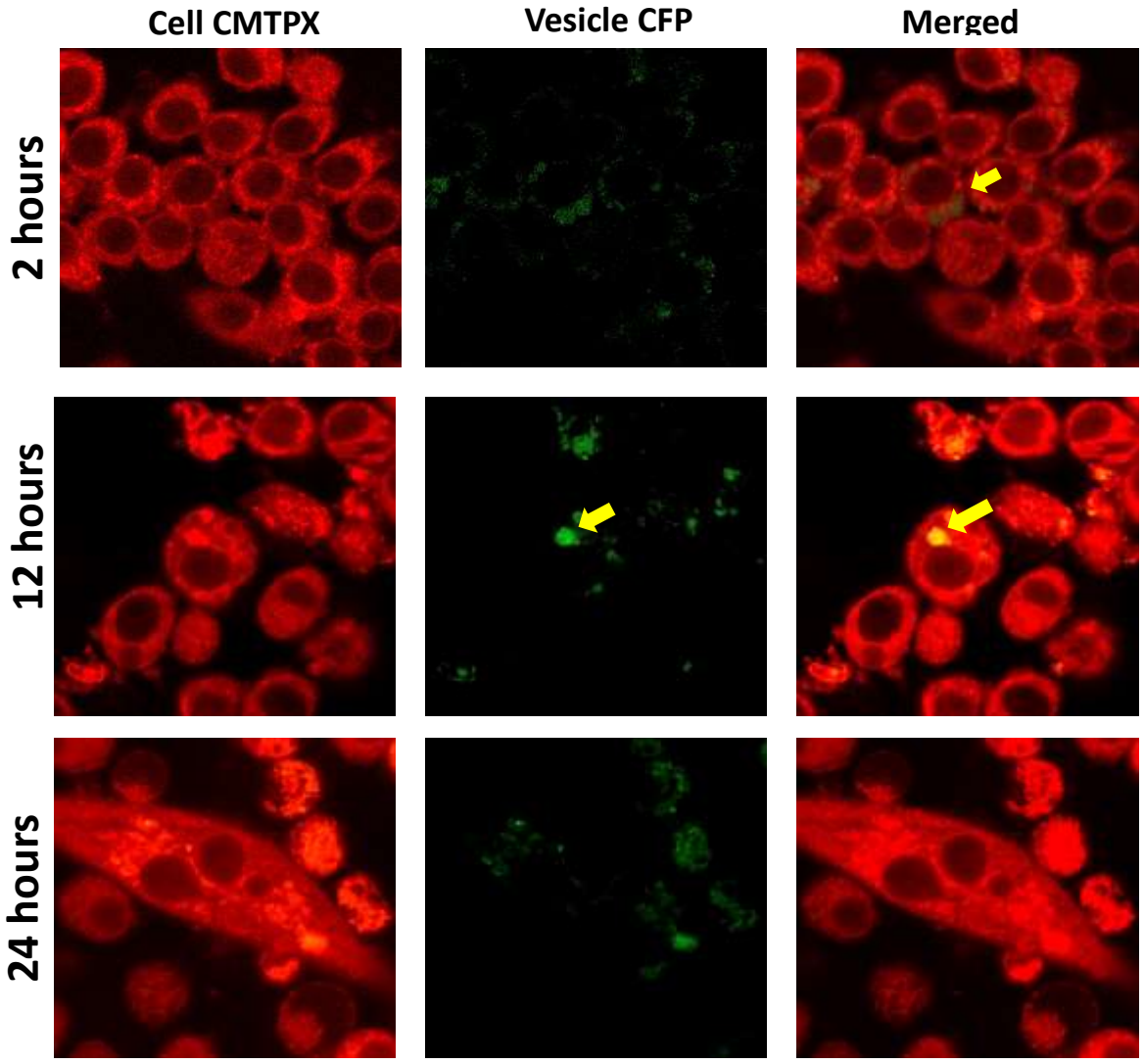


Fig 3.3 Time lapse confocal image of *Y .pseudotuberculosis* MVs associated with murine macrophages. 80% confluent macrophages were prepared on 8 chambered cover glass. Before infection macrophage was stained with CMPTX red. For visualization of MVs 20µg (40 µg/ml) purified MVs from CFP expressing YPIII (+) were treated with macrophage cells for 2 hours. After two hours, unattached MVs were removed by washing with BPBS then subsequently added fresh media visualized under a microscope at different time points Arrow indicates the Localization of vesicles (scale bar 5µm).

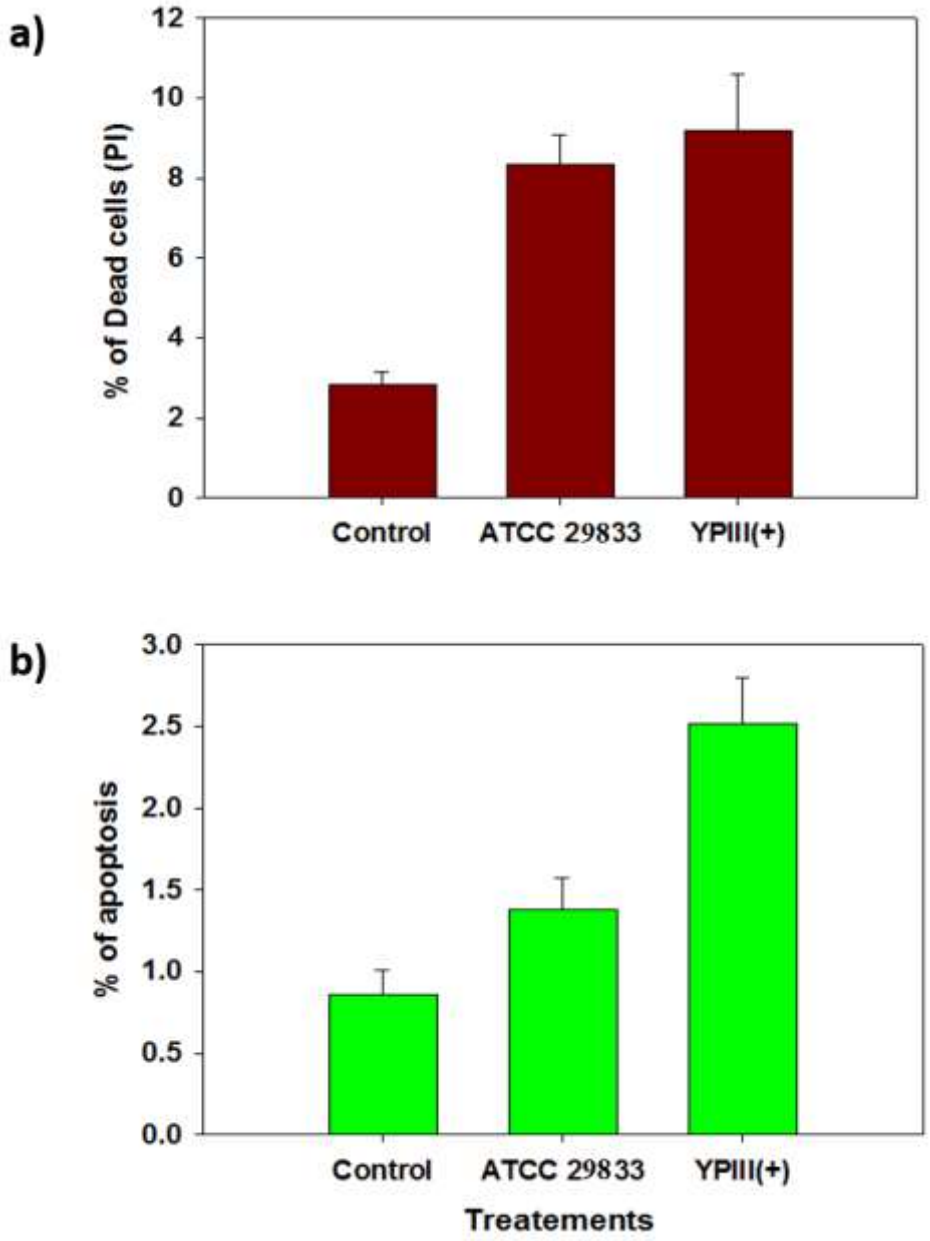


Fig 3.4 FACS analysis of macrophages treated with *Y. pseudotuberculosis* MVs to confirm apoptosis. For apoptosis assay macrophages were treated with 1 ml purified MVs in DMEM F12 in 10 µg/ml media For 24 hours at 37ºC media without MVs used as a control. After 24 hours, of treatment cells were trypsinized and washed twice with Dulbecco's phosphate-buffered saline (DPBS). $1X10^5$ suspension cells in 100 µl Annexing V binding buffer were added to 5 µl of Annexing V and PI were added and incubate the tubes in the dark for 15 minutes at the room.

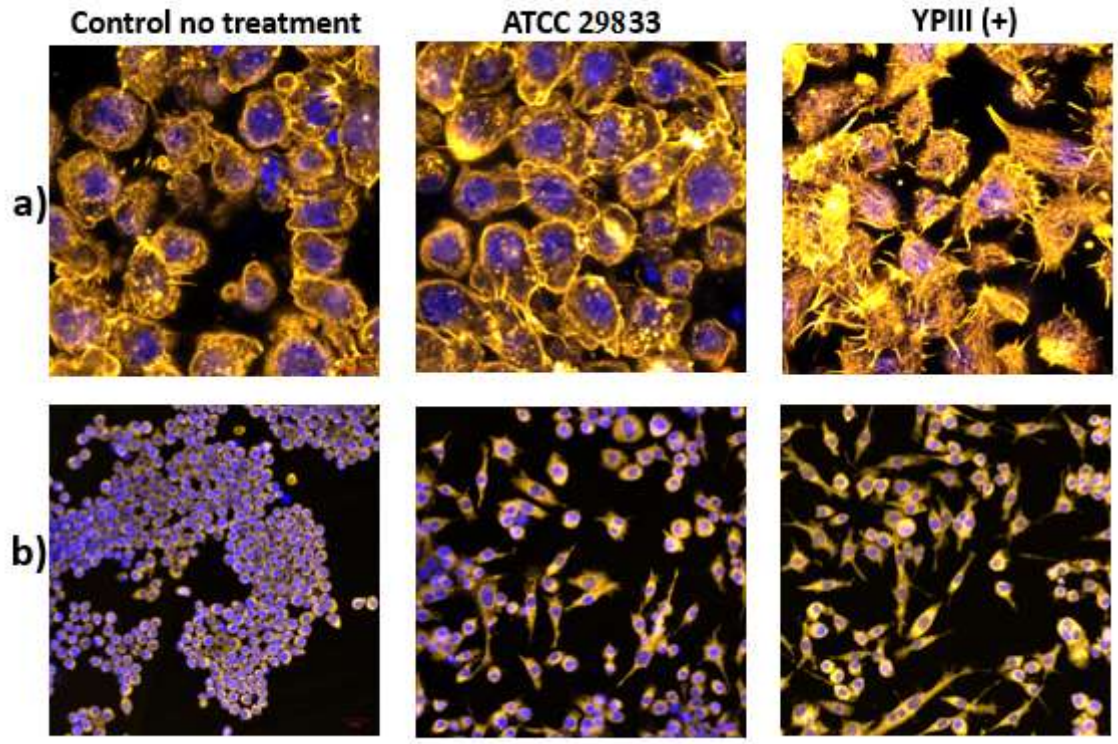


Fig 3.5 F-actin rearrangement of macrophages exposed to membrane vesicle. Macrophages were prepared on 8 chambered cover glass by seeding 25000 cells /well on 8 chambered cover glass. Before treatment of MVs macrophage was serum starved for at least 20 hours after that Vesicle from control without treatment and ATCC29833 and YPIII (+) respectively imaged at 40X magnification. (**b)** macrophage stained with CMTPX red then treated with 10µg/ml of vesicle then incubated 48hour the cells were washed with DPBS and counter stained with DAPI then visualized Unde confocal microscope image were taken at a magnification of 10X.

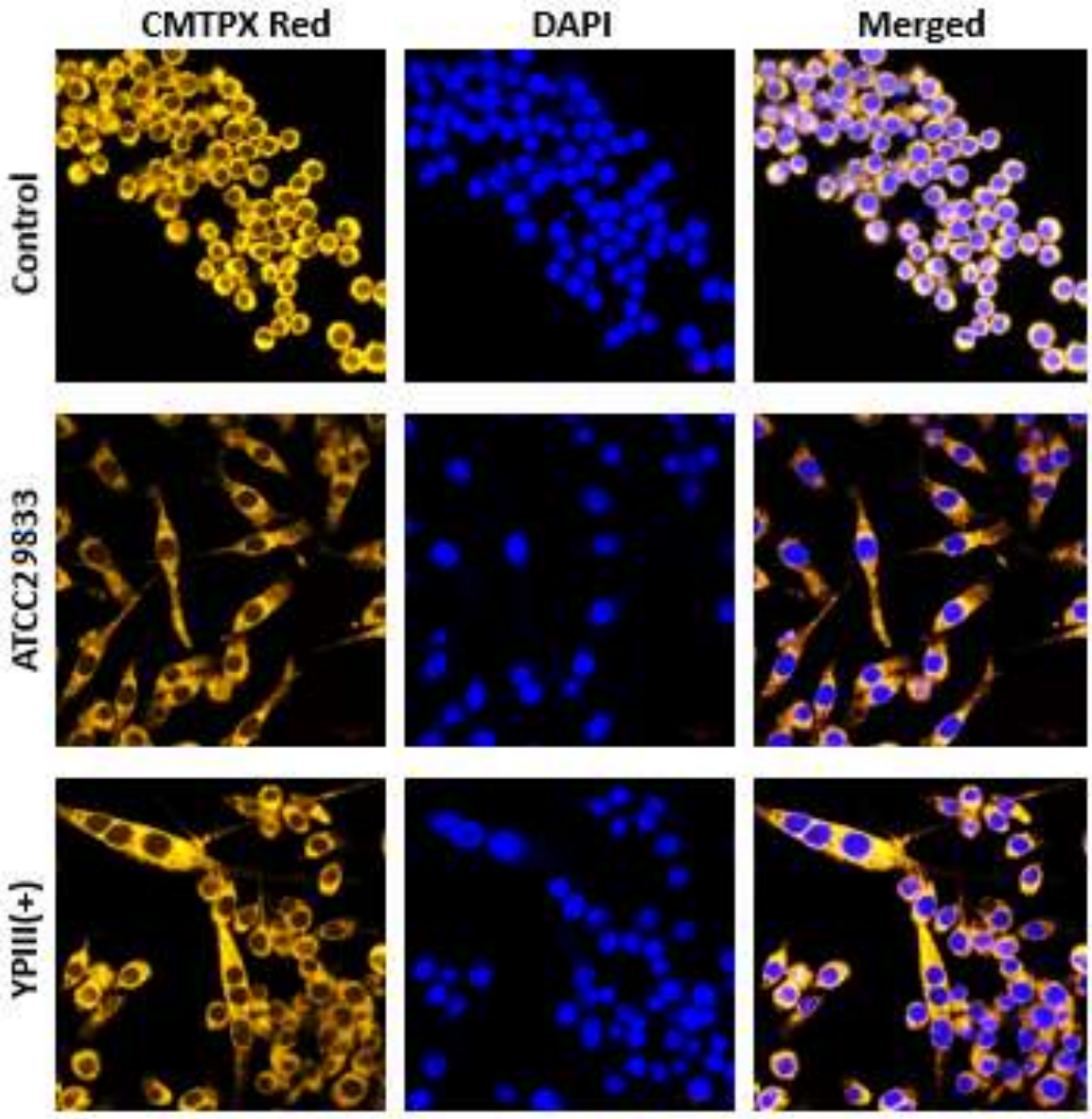


Fig 3.6 *Y. pseudotuberculosis* membrane vesicle leads to morphological changes and multinucleation of macrophages. 80% confluent macrophages were prepared on 96 well plate by seeding 25000 cells /well. Before treatment macrophage was stained with CMPTX red then treated with 10µg/ml of purified MVs from both ATCC29833 and YPIII (+) were treated after treatment cells were incubated for 48 hours. After 48 hours, unattached MVs were removed by washing with DPBS then subsequently fixed with 3.7% of paraformaldehyde incubated 10 min and washed again with DPBS. Nucleus were counterstained with DAPI and visualized under a microscope (scale bar 20 µm).

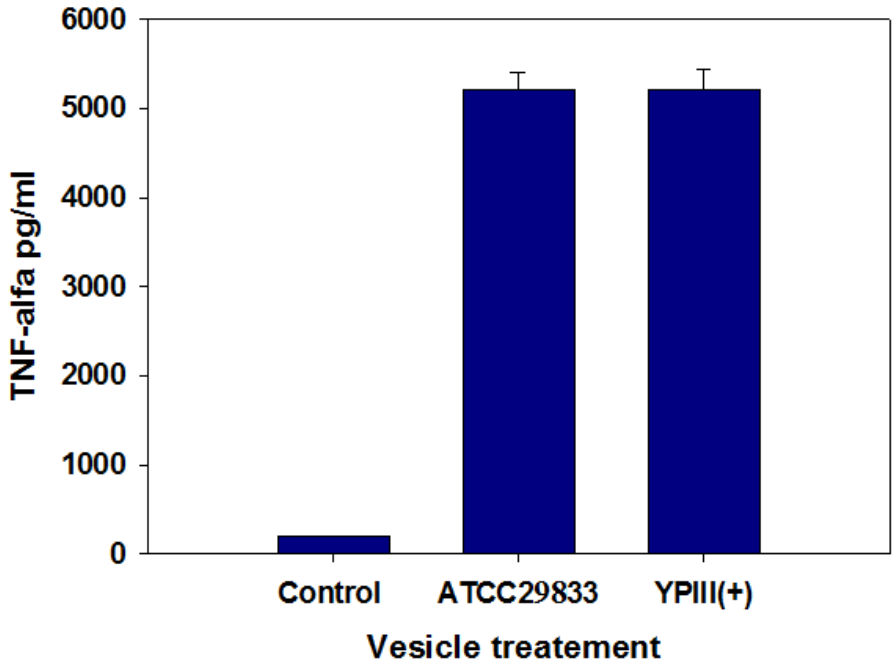


Fig 3.6 Secretion of TNF-alfa by macrophages response to *Y. pseudotuberculosis* MVs. Macrophages cells were grown on 12-well plate $5X10^4$ cells/well for 24 hours, and then replaced media with vesicle 10 µg/ml of MVs (5 µg/well) and incubated in a CO_2 incubator at 37ºC for 24 hours. After 24 hours, cell culture media were collected for ELISA. TNF-α concentration was quantified using ELISA kits from R&D Systems.

3.5 Conclusion

In summary, the data presented in this study focused on the effect of *Y. pseudotuberculosis* membrane vesicle MVs on murine macrophage. Purified membrane vesicles were uptake by the macrophage. The MVs showed cytotoxicity and induced a higher level of the TNF-alfa, and cytotoxicity was not due to the apoptosis. Concluded that the cytotoxicity of the macrophages was due to various proteins associated with the membrane vesicle, and the MVs were shown to be phagocytized by the macrophage. It confirms the presence of the *Yersinia* cytotoxic necrotizing factor CNFy associated with MVs which leads to actin rearrangement and giant cell multinucleation. We hypothesize the novel mechanism of delivery of CNFy into macrophages by a *Y. pseudotuberculosis* though receptor for attachment or secretion system for CNFy not yet identified.

Chapter 4

Analysis of Post Translational Modifications (PTMs) *Yersinia pseudotuberculosis* Cytotoxic Necrotizing Factor (CNFy)

4.1 Summary

Cytotoxic necrotizing factor toxin (CNF) is one of the crucial virulence factor allied with *Yersinia pseudotuberculosis* YPIII. CNFy plays a critical role in the delivery of virulence factor and survival in the host system. Our previous proteomics study we found that there was some signature of post translational modification (PTM) in CNFy protein. For further confirmation, we cloned the ORF of CNFy with its promoter into a pET31b+ vector with His-tag and expressed in both *E.coli* (ECNFy) and *Yersinia pseudotuberculosis* YPIII (YCNFy). Expressed protein were purified by affinity chromatography using Ni-NTA agarose and quantified by ELISA using HisProbe-HRP. Furthermore, 1ug/ml each ECNFy and YCNFy were tested for their multinucleation activity on airways epithelial NuLi-1 cells, result confirms YCNFy is 8 times more active compared to ECNFy. Furthermore purified protein were analyzed by mass spectrometry (LC-MS/MS) followed by post translational modification(PTM) analysis. Surprisingly there were 60 PTMs in the protein YCNFy, but ECNFy had 6 modified amino acid. All six modifications change are common in both YCNFy and ECNFy in that lysine 1003 is methylation one of the specialized PTM found in both protein. There is 17 glutamic acid methylation found in YCNFy. 6 and 37 Dimidiated asparagine found ECNFy and YCNFy respectively.The result suggests that the activity CNFy is interrelated with the post translational modification.

4.2 Introduction

Cyto toxic necrotizing factor **(**CNF) is a pore-forming toxin that was first identified in *E.coli* [107] it was later identified in other bacteria including *Yersinia pseudotuberculosis*[70]. *Yersinia pseudotuberculosis* cytotoxic necrotizing factor (CNFy) gene is encoded by an open reading frame of 3,045bp and located on the chromosomal DNA. The amino acid sequence of CNF exhibits about 61% similarity with *E. coli* CNF1[108]. A recent report about host immune response confirmed that there was a reduction of phagocytosis in the presence of CNFy[109]. The absence of CNFy allows immune cells to rapid elimination of the pathogen. CNFy toxin leads to the formation of actin-rich membrane ruffles and filopodia[110], and also helps to deliver type III effector into epithelial and immune cells *in vitro* also enhance the infection further demonstrated that CNF increases suppression of the antibacterial host response[69]. A Recent study identified the Lutheran (Lu) adhesion glycoprotein/basal cell adhesion molecule (BCAM) as a cellular receptor for CNF[111]. But the receptor for CNF has not been identified. After binding to its receptor, CNF enters endocytic vesicles by receptor-mediated endocytosis, which is independent of clathrin and sphingolipid-cholesterol-rich membrane micro domains (lipid rafts)[112].

The activity of CNF is interesting. CNF has conserved C-terminal catalytic domain between 720 to 1014 amino acid. C-terminal catalytic part of. Cysteine 866, and Histidine 881 have been found to be crucial for the catalytic activity of CNFy. This domain was conserved in the crystal structure, where the third residue of the catalytic triad, Valine 833, was identified[113].

CNF selectively activates RhoA GTPase compared to CNF1. Cell-free lysate supernatants and spent media of *Y. pseudotuberculosis* strains have been shown to contain the complete CNF protein and have induced the development of Hep-2 cells into a giant, multinucleated cells[70]. The activity of CNF is independent of the type III secretion system. The mutinlucealtion is mainly due to the RhoA GTPase activation as CNF catalyzes the deamidation of RhoA at Gln63. This is the one of the post-translational modifications that affect several downstream signaling events, unlike other bacterial toxins[113].

Cell signaling networks are information-processing circuits that control almost all cellular functions. Signalings are regulated by reversible protein post-translational modifications (PTMs)[114] that allow cells to respond rapidly to internal and external cues. Hundreds of different PTMs have been identified. Controlling the spatial distribution of proteins is an important mechanism for regulating their function, and subcellular localization of many transcription factors is extensively regulated by PTMs[115]. Positively charged lysines are often found in the subcellular localization signaling domains of proteins[116], and their acetylation can regulate the subcellular localization of the proteins[109].

PTM is the widespread strategy used by prokaryotes and eukaryotes to modify quickly, locally and specifically the activity of key factors and enable cells to respond rapidly to environmental changes[117]. The diversity of protein functions is generated by PTMs that alter the structure/function relationship and impact protein complex formation, enzyme catalysis and other biomolecule interactions[118]. Recently, the study of PTM in bacterial protein has demonstrated that prokaryotes are capable of modifying proteins with an extensive array of PTMs and that these have a profound influence on bacterial physiology and virulence[119].

Based on a previous study with proteomic analysis of *Yersinia pseudotuberculosis* membrane vesicles we found that some modification may found in the CNFy. The vesicle contains a large quantity of other protein and it was stiffer to confirm the PTM. This study demonstrated the difference in the activity of CNF expressed in *E.coli* (ECNFy), and Yersinia (YCNFy) , and PTM analysis of both proteins showed CNF expressed in *Y. pseudotuberculosis* (YCNFy) has specialized PTM compared to the ECNFy is significantly high in number, here we hypothesized that PTM is required for the activity the CNF protein.

Materials and method

4.3.1Culture of human airway epithelial cell line (NuLi-1)

Bronchial Epithelial NuLi-1(ATCC CLR 40110) cells cultured in T75 flasks in Bronchial Epithelial Cell Growth Medium (BEGM; Lonza USA) substituted with antibiotic (Normocin, 100 µg/ml) until they were confluent. Cells were then washed with sterile DPBS and trypsinized until detached from the flask. Cells then washed with Complete media supplemented with FBS (ATCC, USA), counted and diluted appropriately to seed in an appropriate culture dish for the experiment.

4.3.2 Cloning of CNFy ORF with promoter

CNFy gene with promoter amplified by PCR from genomic DNA of *Y. pseudotuberculosis* strain YPIII using CNF-F 5′-GGATCCATGAAAAATCAATGGCAACATCAATATTT-3′ as sense primer andCNF R 5′-CCCGGGGATATCTTTTCATTTCCCCCTGCC-3′ as an antisense primer, thereby introducing restriction sites for restriction enzymes Xba1 and Xho1 The resulting PCR product was cloned into the expression vector pET31b+ and transformed into *E. coli DH5α* further the plasmid transformed into *Yersinia YPIII* strain.

4.3.3 Expression and purification of His-tagged CNFy

CNFy toxin purification, Both *E. coli* Dh5α and, *Y. pseudotuberculosis* YPIII carrying pET31b-CNF-His grown in LB medium to reach OD 1.0 cells collected by centrifugation and resulted in the pellet were suspended in lysis buffer(50 mM Tris-HCl pH 7.5; 200 mM NaCl) then supplemented with lysozyme 1mg/ml. Suspension kept in ice for 30 minutes before sonication then sonicated, sonicated suspension were centrifuged and purified by affinity chromatography on Ni-NTA agarose (Qiagen; USA).loaded beads were washed with lysis buffer (with 20mM imidazole) at 4 °C. The CNF-His fusion proteins were eluted from the beads by glutathione (500mM imidazole with lysis buffer) twice for 10 min at room temperature.

4.3.4 Quantification of CNFy by ELISA

A standard curve for ELISA was made using CNFy expressed in Yersinia pseudotuberculosis by confirming their purity by SDS page. Purified CNFy were coated on co-star plate using (Coating Buffer: 0.1M sodium bicarbonate buffer; pH 9.6) Incubated for two hours at room temperature then blocked by adding 200µL of blocking Buffer the buffer supplemented with skimmed 5% Milk to each well. Incubate for 30 minutes at 37°C. Washed plate three times with 200µL of wash buffer. Add 100µL of HisProbe-HRP dilutions. Working solution (1:5000 dilution in blocking buffer) to each well and incubate for 15 minutes at room temperature. Washed plate four times with 200µL of Wash Buffer. Added colour reagent A and B (R and D Systems, USA) incubated 30 min at room temperature then stop solution added and allow colour then measure absorbance at 450nm

4.3.5 Quantification of multinucleation

To determine the multi-nucleation, 50% confluent monolayer of NuLi-1 cells prepared in 96 well plates.prior to CNFy treatement cells were stained with CMPTX Red (life technologies, the USA). After staining different concentration of ECNFy and YCNFy treated to the stained cells and incubated for 48 hours in a CO_2 incubator with 5% CO_2 were stained with CMTPX red then incubated with the. After incubation cells were washed with DPBS (Sigma-Aldrich, USA) and fixed with 4.0 % of paraformaldehyde for 10 min at room temperature. Fixative were removed by washing three times with DPBS and by washing, and fixed cells were counterstained with DAPI. Then cells were visualized under the confocal microscope (LSM 700: Carl Zeiss, Germany) at the magnification 20X and images captured using Zen 2009 software.Multinucleated cells were visually counted and calculated percentage of multinucleated cells.

4.3.6 MTT assay to assess cytotoxicity of ECNFy and YCNFy

The toxic effects of purified CNFy in-vitro were analyzed by quantitatively determining cell viability of human airway epithelial cell (NuLi-1). In 96-well plate, 50% confluent airway epithelial (HAE) was prepared and treated with 2000 ng down to 20 ng of ECNFy and YCNFy in BGEM media and incubated for 48 hours in a CO2 incubator. Briefly, MTT solution (5 mg/ml) in BGEM media was added to each well in an amount equal to 10%. Cells were incubated for at least 2 hours at 37°C.after that the media were removed, and cells were lysed with DMSO (Sigma-Aldrich, USA) Absorbance was measured at a wavelength of 540 nm, and the percentage of viable cells was calculated as the ratio of absorbance of stained cells.

4.3.7 Analysis of CNFy Proteins by LC-MS/MS

SDS-PAGE gel electrophoresis analyzed purified CNF proteins. 5ug microgram CNFy from both preparation were suspended in loading buffer (62.5 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% (v/v) glycerol and 0.001% bromophenol blue) and denatured by heating at 95 ºC for 5 min. Samples loaded onto a 10 % SDS-PAGE. After electrophoresis, the gels were washed with water then stained with colloidal Coomassie.

For tryptic peptides preparations (5 μg) were applied to 7.5 % Tris-glycine SDS-PAGE gel (Bio-Rad, USA). After staining with colloidal Coomassie, the each band separated into 6 were subjected to in-gel tryptic digestion, as described by Shevchenko and co-workers [85]. The resulting tryptic peptides were analyzed by LC-MS/MS. All mass analyzes were performed on an LTQ-Orbitrap (Thermo, Bremen, Germany) equipped with a nanoelectrospray ion source. To separate the peptide mixture, we used a C18 reverse phase HPLC column (150mm x 75um

i.d.) using an acetonitrile/0.1% formic acid gradient from 10 to 24% for 90min at a flow rate of 300 ml/min.

For MS/MS analysis the precursor ion scan MS spectra (m/z 400–2000) were acquired in the Orbitrap at a resolution of 60,000 at m/z 400 with an internal lock mass. The 20 most intensive ions were isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID).All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.1.14) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest and X! Tandem were set up to search the *Yersinia pseudotuberculosis* YPIII CNFy (http://www.uniprot.org/)) assuming the digestion enzyme trypsin.

Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Sequest and X! Tandem as a fixed modification. Deamidated of asparagine and glutamine, methyl of glutamic acid, lysine, glutamine and arginine, oxidation of methionine, acetyl of lysine, serine, threonine and the n-terminus and phosphor of serine, threonine and tyrosine were specified in Sequest and X! Tandem as variable modifications. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus were specified in X! Tandem as variable modifications.

Scaffold PTM (Proteome Software, Portland, Oregon, USA) was used to annotate PTM sites derived from MS/MS sequencing results obtained using Scaffold (version Scaffold_3.0). Using the site localization algorithm developed by Sean A et al[120] Scaffold PTM re-analyzes Scaffold PTM re-analyzes MS/MS spectra identified as modified peptides and calculates A score values and site localization probabilities to assess the level of confidence in each PTM localization. Scaffold PTM then combines localization probabilities for all peptides containing each identified PTM site to obtain the best-estimated probability that a PTM is present at that particular site.

4.4 Result and discussion

4.4.1 CNFy expressed in *Y. pseudotuberculosis* and *E.coli*

Initially, the CNFy was cloned into PET 31b+. To control the expression level, we used native promoter of CNFy. The promoter showed compatible in *E. coli,* but the expression level was very low. Further, CNFy was expressed in *E. coli* (ECNFy), and *Yersinia pseudotuberculosis.* (YCNFy) Purified protein was analyzed in SDS-PAGE gel and stained with colloidal Coomassie and confirmed the expression of CNF with a clear band observed around 115 kDa in both hosts. The concentration was determined by ELISA using a His-probe HRP conjugate Kit. The concentration of ECNFy and YCNFy was 122ug/ml and 276ug/ml, respectively. The concentration variation was clearly visible in SDS page gel. **(Fig 4.1a)**

4.4.2 Activity of CNFy expressed in *Y. pseudotuberculosis* YPIII (+) was higher compared *E. coli*.

Various studies have shown that CNFy is one of the toxins that leads to multinucleation in a different type of cells[108]. In our previous study, complementation of CNFy in *Yersinia pseudotuberculosis* ATCC 29833 showed multinucleation in NuLi-1 cells **(Fig 4.2)**. For further confirmation, the purified CNF from both hosts were tested on human airway epithelial cells NuLi-1 cells. CNF from both preparations was quantified by ELISA, and prepared final concentration 1000ng/ml then diluted serially and used for the multiplication test. The result confirms YCNFy leads to the formation of more than 80% multinucleation and even 500ng/ml also showed same activity, suggesting that the concentration more than 500ng optimal concentration. But ECNFy showed much lower levels, around 12% of multinucleated cells observed after treatment. The result suggests that the protein is 8- to 10-fold less potent in triggering multinucleation compared to YCNFy (**Fig 4.4 and 4.2**). The result is acceptable because recombinant protein expressed in different hosts have different activity because of the unfolded or the confirmation of the protein will be compromised when shown in a different host[121].

4.4.3. CNFy is nontoxic to human airway epithelial cells

To determine the adverse effect of CNF on human airway epithelial cells. The viability of airway epithelial cells after 48 hours of treatment with purified ECNFy and YCNFy was determined by MTT assay. CNF were treated with Different concentration to the 50% confluent cells assay treated with ECNF and YCNF to the airway epithelial cells 1000ng/ml to 10ng/ml The results confirm that both preparations of CNF were non-toxic to the airway epithelial cells **(Fig 4.3).** A previous study has shown that I.C.V injection of the entire CNF toxin causes no lethal effects in animals[109].

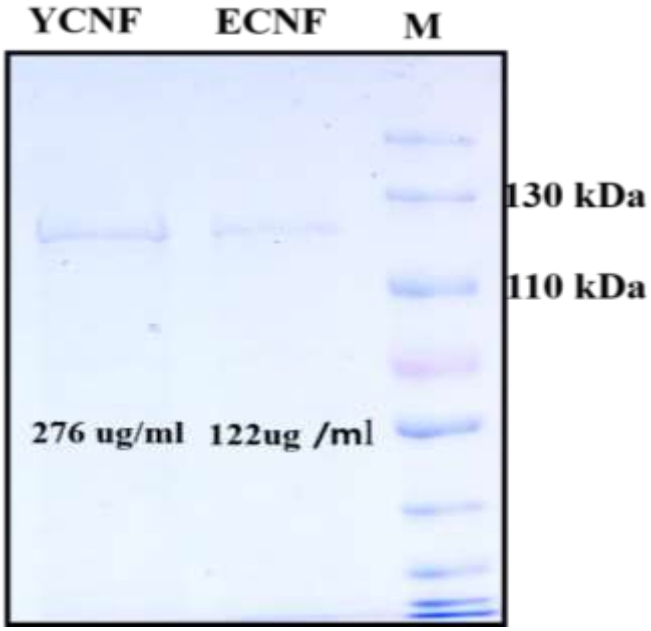


Fig 4.1 The SDS-PAGE analysis of purified CNFy from both and *Y. pseudotuberculosis* YPIII (YCNF) *E. coli* (ECNF). a) *Y. pseudotuberculosis* and *E. coli* grown LB media till optical density Reaches to 1.0 OD the bacteria were pelleted and lysed with lysis buffer and sonicated and using Ni-NTA and purified protein visualized in SDS-PAGE **b)** Quantification multinucleation after treatment of known concentration of CNFy expressed in both hosts.

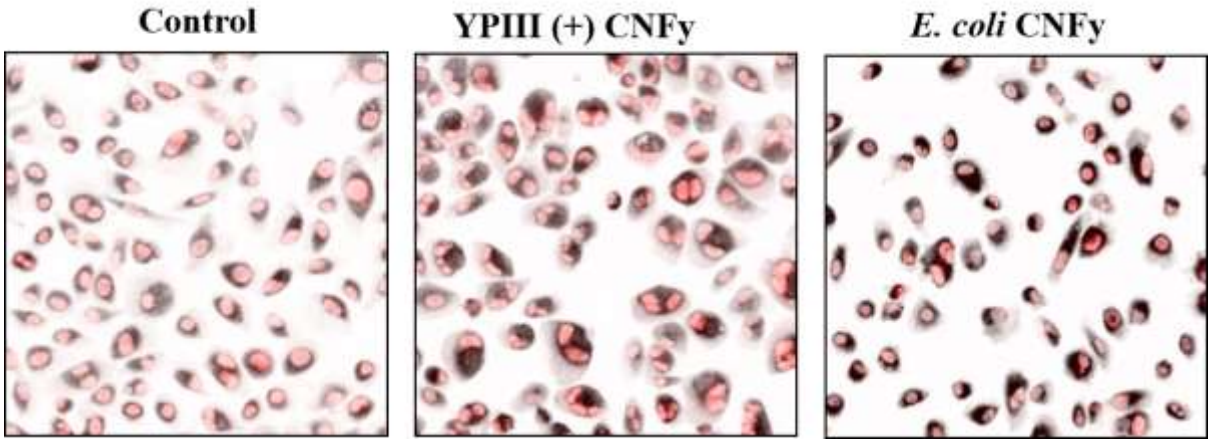


Fig 4.2 Laser confocal image of multinucleated cells. For the quantification of multinucleation CMTPX Red stained NuLi-1 cells were treated with purified CNFy at different concentration and media without CNF as Control. After treatment cell's nucleus were stained with DAPI and imaged under confocal microscope at the 20X magnification (Images represented with false colouring)

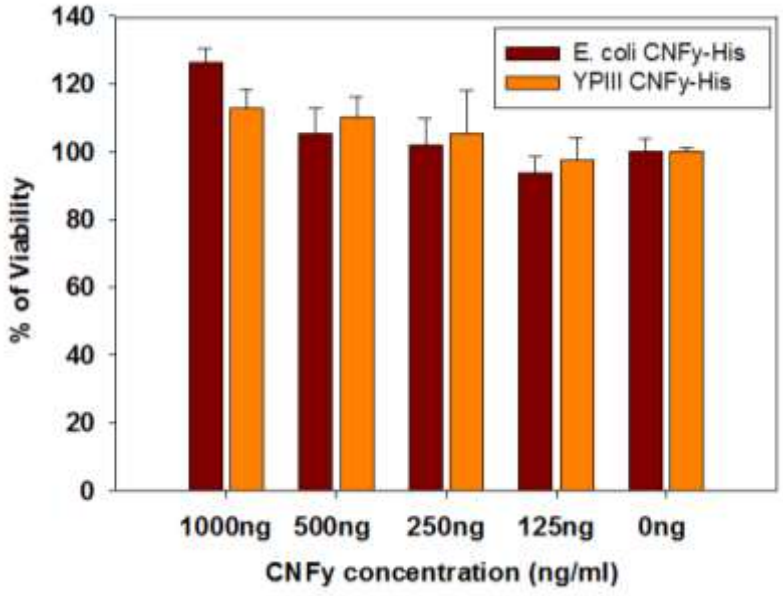


Fig 4.3 Effect of purified CNFy on NuLi-1 Cells. The viability of the NuLi-1 cells was determined by MTT assay after 48-hour post treatment of different concentration of ECNFy and YCNFy.

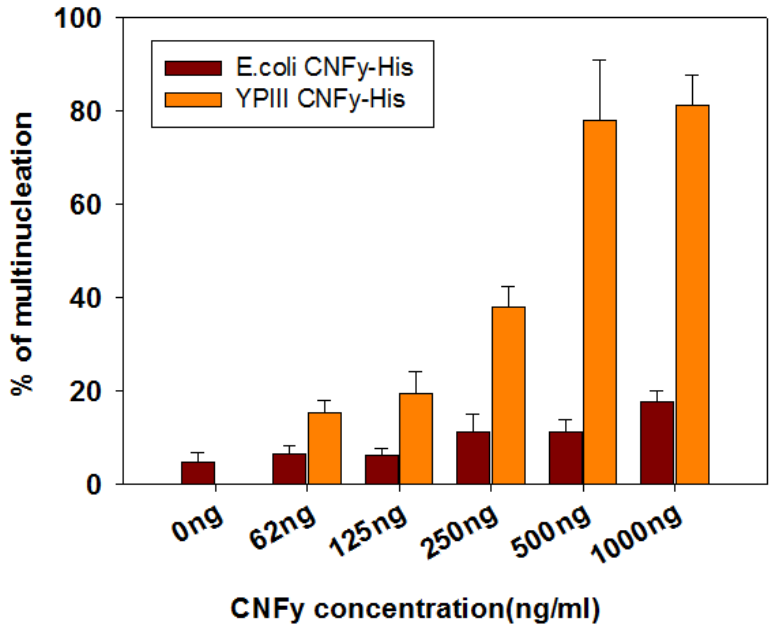


Fig 4.4 Quantification of multinucleation in NuLi-1 cells caused due to purified CNFy treatment. The viability of the NuLi-1 cells was determined after 48-hour post treatment of different concentration of E CNFy and YCNFy.

4.4.4 PTM analysis revealed high level of post translational modification in YCNFy

Mass spectrometry analysis of CNFy protein showed there is a diverse post translational modification (PTM) present in the CNF expressed in both **(Fig 4.5)** *Y. pseudotuberculosis* YCNFy and *E.coli* ECNFy. Compared to YCNFy 60 modifications, ECNFy has only six modification. Out of 6 modification lysine, 1003 is common modification found in ECNFy and YCNFy **(Table 4.1).** Over all YCNFy has diverse post translational modification total of 60 deaminations of the asparagine is one of the dominant modification present in both YCNFy and ECNFy has the highest modification**. (Table 4.2)** methylation of glutamic acid is the second higest post translational modification present in the YCNFy, but it was absent in the ECNFy.

4.4.5 Lysine (K) and glutamic acid (E) methylation in CNFy

Lysine methylation is well characterized. In histone protein, it regulates the function of histone. Methylation is the addition of a methyl group into the amino acid, and the amino acid will have a change in its mass of 14 Da. Methylation of lysine residues in histones in DNA is the main regulator of chromatin structure and transcriptional activity in eukaryotic systems. It depends on some addition and methylation can also be subdivided into two functionally different forms. i) *N*-methylation of Lys/Arg/Gln, and ii) *N*-methylation involves the transfer of either one, two or three methyl groups onto the terminal amine (the number being dependent largely on the residue; the primary amine of Lysine can accept up to three methyl groups[122].

In our study YCNFy has multiple methylations in amino acid 613 and 1003, but the ECNF has methylation in the 1003 amino acid only. Lysine methylation was previously detected in bacteria detected in *L. interrogans* identified 64 lysine-methylated sites from 58 proteins[123]. Methylation is due to the enzymatic reaction by methyltransferases. Previous report some methyltransferase enzyme from *Rickettsia* although could methylate it's at multiple sites catalysed tri-methylation, while mono-, di- and tri-methylation[124]. In *Yersinia,* vagH methyl transferase is necessary for the *in vivo* survival of *Y. pseudotuberculosis*[125]. Also, two more methyl transferases protein related to pathogenicity and they are critical for disease progression. Lysine methylation also plays a role in receptor-mediated signal transduction[125].

In this study, we found that methylation of glutamic acid is the second largest type of PTM. There are 27 glutamic acids methylated amino acid found in YCNFy. Interestingly it was not detected in ECNFy. Previous studies have reported glutamic acid modification in *E.coli* protein, and it has been shown to modulate chemotactic responses in *E.coli*[126]. It was hypothesized that methylation of glutamic acid plays a key role in protein folding and functions; it is expected that neutralization of the amino acids, negative charge would have a significant impact on the proteins[127]

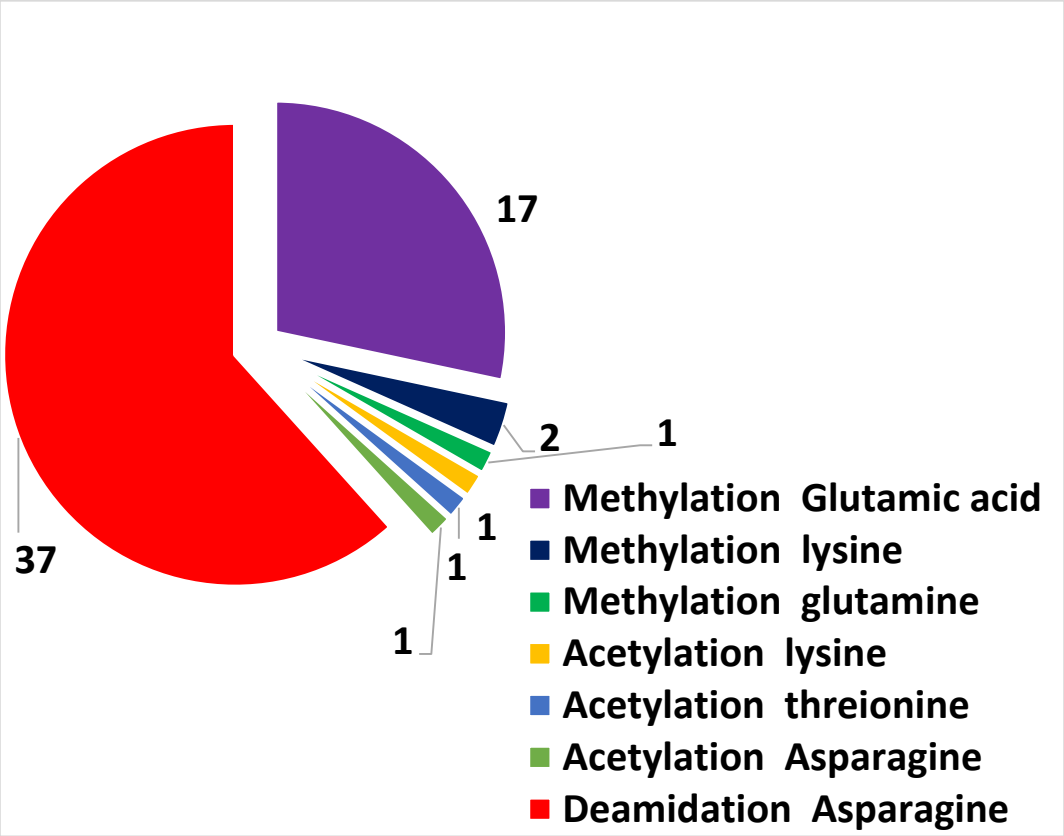


Fig 4.5 various post translational modification (PTMs) present in CNFy expressed in *Y. pseudotuberculosis* YPIII (+)

4.4.6 Acetylation found only in YCNFy, not in ECNFy

Acetylation is one of the prominent PTM in bacterial protein. Protein *N*-acetylation is the addition of an acetyl group to either the ε-amines of Lys side chains (N^{ε}-acetylation) or the α-amines of protein *N*-termini (N^{α}-acetylation) and it was first identified in eukaryotic histones. In our study, we found acetylation of lysine, threonine, and asparagine was found in only YCNFy **(Table 4.2).** Lysine acetylation is linked with acetyl-coenzyme a metabolism also cellular signaling[128]. Recent research has highlighted the mechanisms by which acetylation regulates various cellular processes.[109] Lysine is one of the most frequently modified amino acids, and it can accommodate several types of acylation. Acetylation also leads to subcellular localisation some of the protein in eukaryotes[129]. Lysis acetylation may help the CNFy to localize in the cells with their interaction partner.

4.4.7 Deamidation of asparagine (N)

Deamidation of the asparagine was found to be most abundant PTM in YCNFy total of 37 amino acids were deamidated it is more than 50% PTMs in YCNFy. Deamidation of asparagine is also associated with ECNFy, but it is a relatively low number **(Table 4.2)** Deamidation is the critical protein post-translational modifications that contribute leads to various disease celiac disease, urinary tract infection, cataract formation, cancer, Alzheimer's[130]. Deamidation also effects the therapeutic protein purity and shelf life. It is the one of the known non enzymatic modification it occurs in both occur both *in vivo* and *in vitro*[131]

Table 4.1 Post translational modification commonly present with CNFy expressed in *Y. pseudotuberculosis* YPIII and *E. coli*

Serial No	Amino acid	Modification
1	N149	Deamidated
2	N451	Deamidated
3	N611	Deamidated
4	N988	Deamidated
5	N1000	Deamidated
6	K1003	methyl

Table 4.2 Post translational modification **(PTMs)** Modification found in different amino acid

Modification	Amino acid	YCNFy	ECNFy
Methylation	Glutamic acid	17	0
	Lysine	2	1
	Glutamine	1	0
Acetylation	Lysine	1	0
	Threonine	1	0
	Asparagine	1	0
Deamidation	Asparagine	37	5

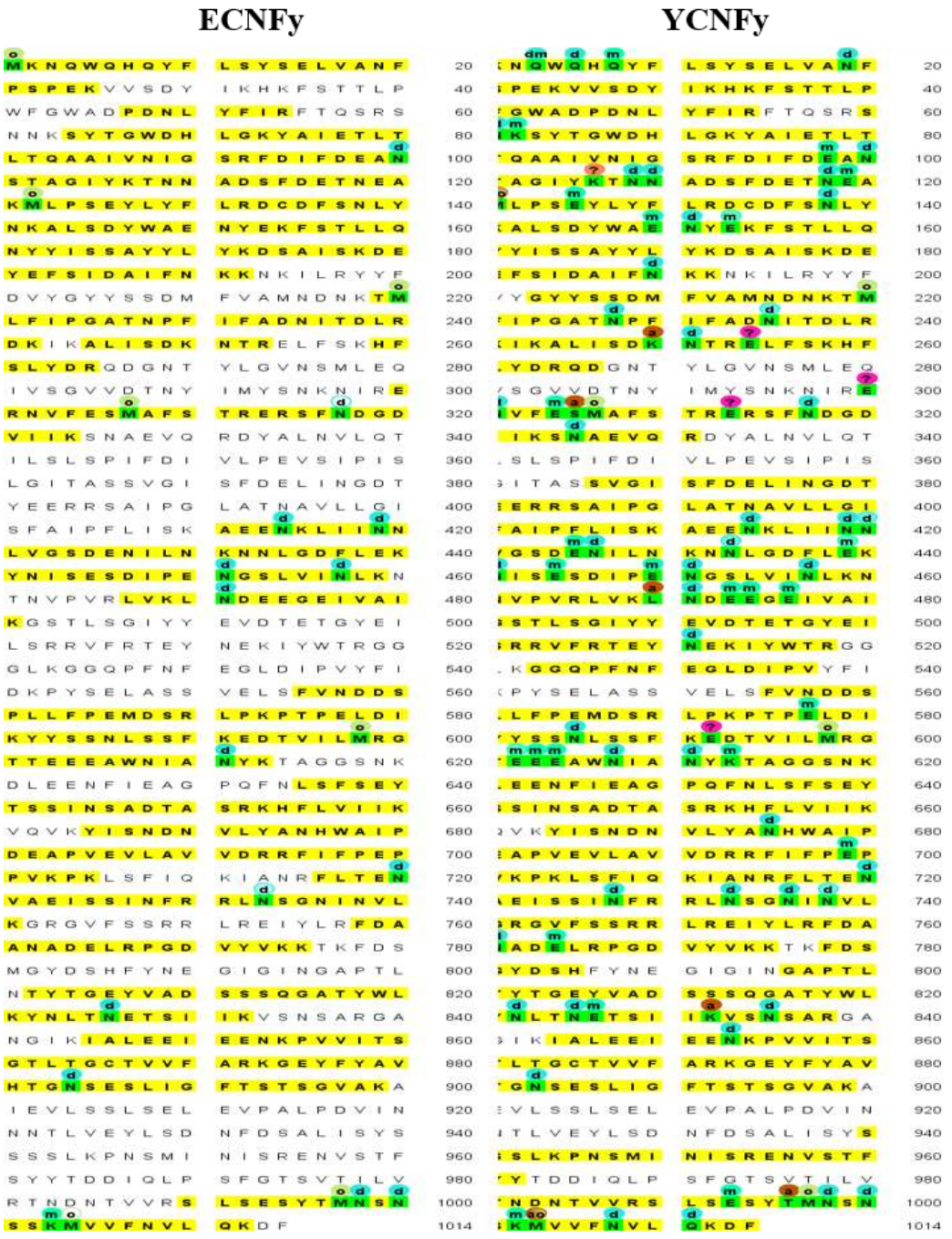


Fig 4.6.Protein sequence data obtained from the Scaffold PTM showing the presence of PTM CNF expressed in *E. coli* (ECNFy), and *Y.pseudotuberculosis* (YCNFy). ECNFy has much lover modification compared to YCNFy.

4.5 Conclusion

Post translational modification (PTM) occurred in cytotoxic necrotizing factor (CNFy); CNFy expressed in *Y. pseudotuberculosis* shown a significantly higher rate of Post translational modification compared to CNFy expressed in *E. coli*. Based on previous reports post translational modification are play a key role in cells signaling during pathogenesis and disease progression, Methylation of lysine (K), glutamic acid(E) and deamination of asparagine(N) and lysine (K) acetylation are characterized in the context of cells signaling or disease progression. In our study CNFy is one of the major virulence factors that causes very precise signaling event during interaction with the host system. It is clearly confirmed CNFy expressed in *E. coli* is less active compared to the YCNFy. So here we hypothesize post translational modification is probable players in the activity of CNFy. Further research should be focused on identification for essential PTM for the activity and identification of enzyme required for the modification in *Y. pseudotuberculosis*.

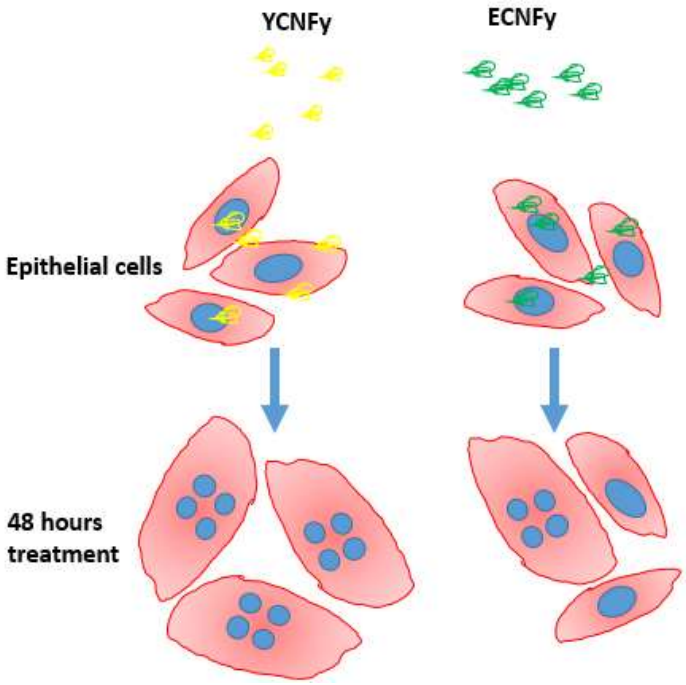


Fig 4.7 Hypothetical model showing YCNFy leads higher level of giant cell multinucleation compared to ECNFy.

Chapter 5
Conclusion

Chapter 2 discussed the production and characterization of *Yersinia pseudotuberculosis* membrane vesicles (MVs) and MV-associated vesicular proteins including various virulence factors. Purified MVs induced morphological changes in human airways epithelial cells NuLi1 and were shown to be cytotoxic when they were internalized by human host cells. MVs delivered cytotoxic necrotizing factor (CNFy) and chaperonin-60 kDa inside NuLi1 cells.

Chapter 3 was more focused on the response of immune cells. The study was continued to the direction of what could be the response on murine macrophages RAW 264.7 cells. Purified MVs from CFP expressing ATCC 29833 and YPIII (+) strains were used to treat murine macrophages until 48 hours. There was a significant loss in viability due to MVs treatment with viability reducing down to around 50%. Also giant cell multinucleated phenotype were observed after exposure to MVs. A Higher level of TNF-α from Raw cells were induced after 24 hours of exposure to MVs from both ATCC and YPIII strain (≥5.2 ng/ml). Further study confirmed there was no significant apoptotic population among the MV treated raw cells. This suggested that apoptosis was not a major event in MV mediated cytotoxicity in mouse macrophage cells.

Chapter 4 we focused on characterizing Cytotoxic necrotizing factor toxin (CNFy), which is one of the major virulence factor associated with *Yersinia pseudotuberculosis* YPIII. CNFy plays a crucial role in delivery of virulence factor and survival in host system. In our initial proteomics study we found signature Post translational modification of the *Yersinia pseudotuberculosis* CNFy protein. For further confirmation, CNFy was expressed in *E.coli* and YPIII strains and purified CNFy were quantified using ELISA using His probe HRP conjugate. 1ug/ml of purified CNFy was tested for multi-nucleation in NuLi1 cells. Results suggested that CNFy expressed in *Yersinia pseudotuberculosis* YPIII (+) has 10 times more activity than CNFy expressed in *E.coli.* And when the purified protein was further subjected to post translational modification (PTM) analysis using mass spectrometry, we surprisingly found 60 Post translational modifications in the protein expressed in *Yersinia pseudotuberculosis* YPIII while PTM in *E. coli* CNFy revealed only 6 amino acid modifications. Out of these six modifications, methylation in Lysine 1003 was found common in both *Yersinia* and *E.coli*. In contrast, 17 glutamic acid methylations could be detected in CNF expressed in *Yersinia pseudotuberculosis* only.

Future prospective

This study lay down a foundation for the application of membrane vesicles: a potential candidate for the acellular vaccine. *Yersinia* is known to be one of the candidates for killed vaccine.Yersiniavax® is the one whole cell killed vaccine that made use of inactivated *Yersinia pseudotuberculosis* and is used for the immunization of wild deer. In our study, we found that 60 kDa

Chaperonin is the most abundant and constantly concentrating protein associated with membrane vesicle. It is one of the pathogenesis-related protein in most of the pathogens. Chaperonin 60 proteins directly activate cells that lead to the inflammatory response. Chaperonin is on of the candidate for the subunit vaccine.These properties were explored in *B.pertusis* subunit vaccine made from the bacterial chaperonin 60 proteins. Furthermore, the recombinant chaperonin also concentrated in the In *E. coli.* So, further study should be targeting chaperonin to control the *Yersinia infection*.

Bacterial toxins play an important role in infection and disease pathology. At the same time, some toxins can serve as potential candidates for therapeutics due to their immune inducing property. CNF1 from *E. coli* has already been under consideration for this scheme. *In vivo* Studies revealed that intravenous injection CNF1 was not lethal for mice, but its *in vivo* application was difficult due to high molecular weight (114kDa). In our study we showed that CNFy from *Yersinia Pseudotuberculosis* was 65% similar to CNF1 and particularly activated RhoA showing same activity and having a lower molecular weight. So we propose and expect to elaborate our study relating CNFy to explore its alleged therapeutic potential. Altogether Chaperonin 60 and CNFy from *Y. Pseudotuberculosis* offers an intriguing clue for future vaccination and therapeutic strategy. This can open up the scope of new research in future.

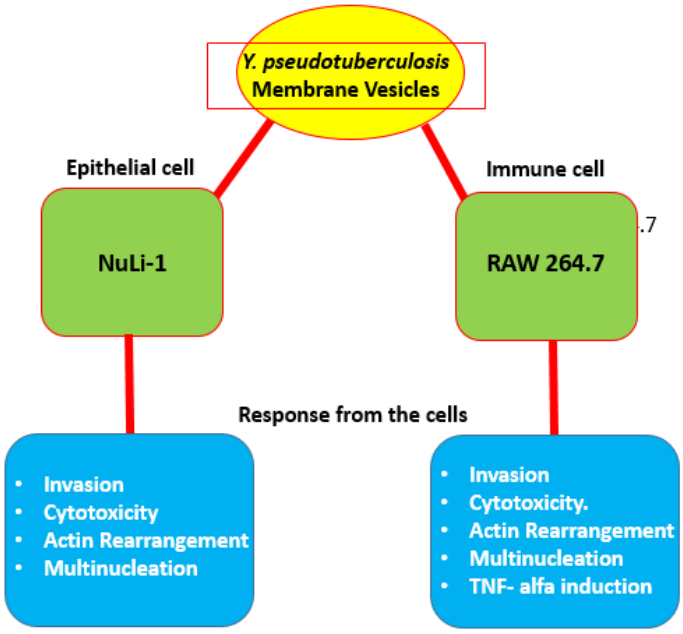
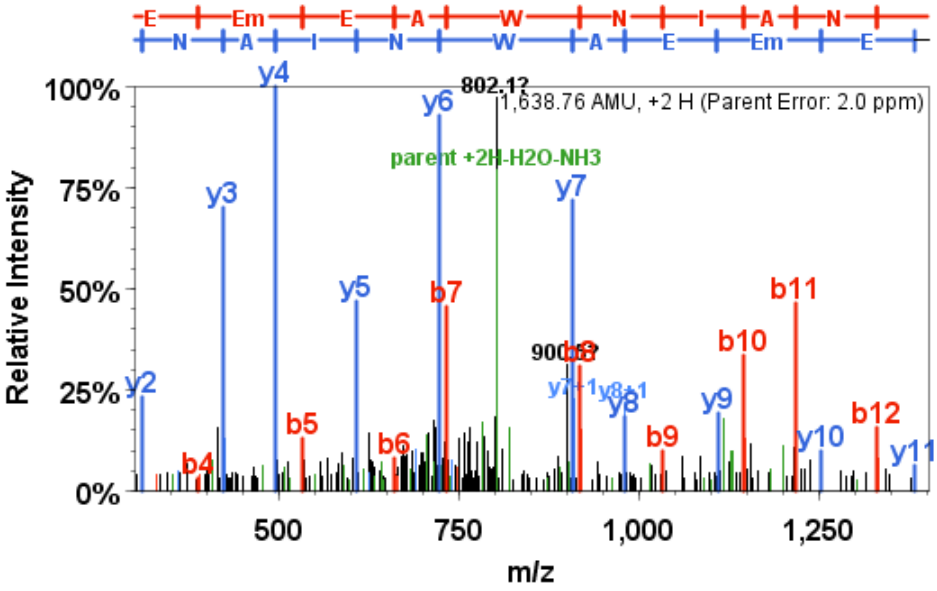
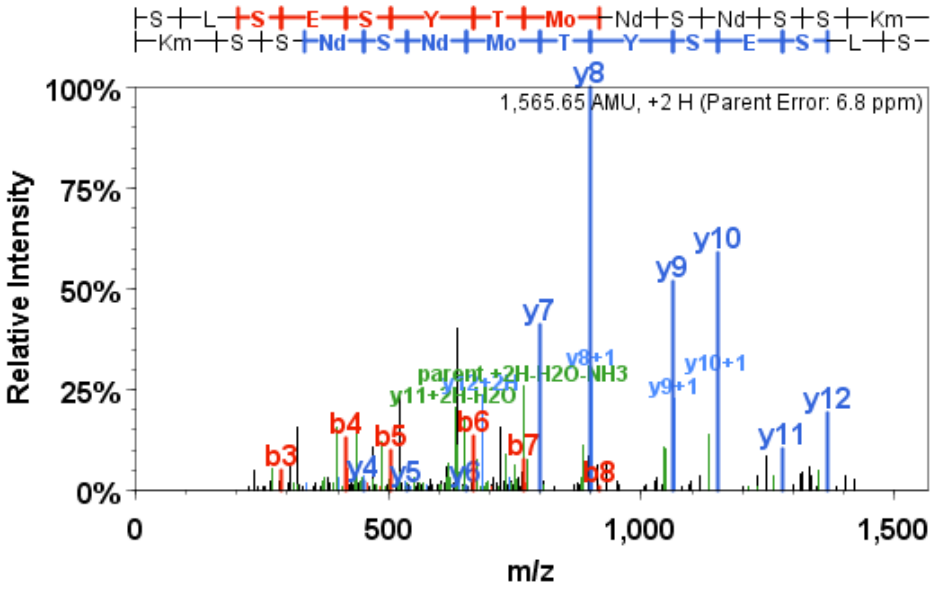


Fig 5.1 *Yersinia pseudotuberculosis* membrane vesicle interact with different host cells and series of response from the host.

APPENDIX -1 Representative mass spectrum of glutamic acid (E) Methylation and Lysine(K) Methylation



1) Glutamic acid methylation (E 605) in cytotoxic necrotising factor



2) Lysine methylated peptide (K 1003) methylated in both YCNFy and ECNFy

ACKNOWLEDGEMENTS

I would like to thank my Ph.D. advisors, Professor Robert James Mitchell for supporting me during these past five years. Professor has been supportive and has given me the freedom to pursue various projects without objection. He has also provided insightful discussions about the research. I also have to thank the members of my Ph.D. committee Prof. Cheol-Min Ghim, Prof. Hyung Joon Cha, Prof. Sang Sun Yoon, Prof. Joon-Hee Lee, Prof. Nam Dougu for their suggestions related to my thesis work. I will forever be thankful to Prof. Jeong Kon Seo, mass spectrometry specialist and my former Advisor Dr. Sukhada Mohandas, Dr. P Paneerselvam, Dr. N. Ramaiah, Dr. K.C Shivananadappa and under graduate advisor, Dr. Raghavendra. Dr. Raghavendra Rao was and remains my best role model for a scientist, mentor, and teacher. Dr. Rao was the reason I decided to go to pursue a career in research. His enthusiasm and love for teaching are contagious.

I also thank my lab mates, Dr. Bari, Seongyeol, Son, Hansol, Aram, Sandy, Dr. Siseon, and Dr.Mohammed and beside labmates Dr.Vinu and Dr.Sathish.

Special thanks to my wife Ghana and my daughter Adhitri Kaveramma, who all have been supportive and caring. The best outcome from these past five years is finding my best friend, soul-mate. I love them so much, and I would not have made it this far without them.

I also thank my friends (too many to list here but you know who you are!) for providing support and friendship that I needed. I would like to thank Vijay Kumar Modeplalli for being supportive throughout my time here in UNIST.

I especially thank my, dad, Monnappa K.K, mom Pushpavathi K.M and my brother Akshay K M, My hard-working parents have sacrificed their lives for my brother and myself and provided unconditional love and care. I love them so much, and I would not have made it this far without them.

References

1. Breitschwerdt, E. B.; Linder, K. L.; Day, M. J.; Maggi, R. G.; Chomel, B. B.; Kempf, V. A. J., Koch's Postulates and the Pathogenesis of Comparative Infectious Disease Causation Associated with Bartonella species. *Journal of comparative pathology* **2013,** *148* (2-3), 115-125.
2. Hernandez, M.; Dutzan, N.; Garcia-Sesnich, J.; Abusleme, L.; Dezerega, A.; Silva, N.; Gonzalez, F. E.; Vernal, R.; Sorsa, T.; Gamonal, J., Host-Pathogen Interactions in Progressive Chronic Periodontitis. *Journal of Dental Research* **2011,** *90* (10), 1164-1170.
3. Casadevall, A.; Pirofski, L. A., Host-pathogen interactions: Basic concepts of microbial commensalism, colonization, infection, and disease. *Infect Immun* **2000,** *68* (12), 6511-6518.
4. Casadevall, A.; Pirofski, L. A., Host-pathogen interactions: Redefining the basic concepts of virulence and pathogenicity. *Infect Immun* **1999,** *67* (8), 3703-3713.
5. Carvalho, F.; Sousa, S.; Cabanes, D., How Listeria monocytogenes organizes its surface for virulence. *Frontiers in cellular and infection microbiology* **2014,** *4*, 48.
6. Riddihough, G., First-line defence. *Nature* **1994,** *372* (6501), 114.
7. Yamamoto, Y.; Harashima, A.; Saito, H.; Tsuneyama, K.; Munesue, S.; Motoyoshi, S.; Han, D.; Watanabe, T.; Asano, M.; Takasawa, S.; Okamoto, H.; Shimura, S.; Karasawa, T.; Yonekura, H.; Yamamoto, H., Septic Shock Is Associated with Receptor for Advanced Glycation End Products Ligation of LPS. *J Immunol* **2011,** *186* (5), 3248-3257.
8. Bhakdi, S.; Bayley, H.; Valeva, A.; Walev, I.; Walker, B.; Weller, U.; Kehoe, M.; Palmer, M., Staphylococcal alpha-toxin, streptolysin-O, and Escherichia coli hemolysin: Prototypes of pore-forming bacterial cytolysins. *Archives of microbiology* **1996,** *165* (2), 73-79.
9. Ribet, D.; Cossart, P., How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect* **2015,** *17* (3), 173-183.
10. Miyoshi, S., Extracellular proteolytic enzymes produced by human pathogenic Vibrio species. *Front Microbiol* **2013,** *4*.
11. Costa, T. R. D.; Felisberto-Rodrigues, C.; Meir, A.; Prevost, M. S.; Redzej, A.; Trokter, M.; Waksman, G., Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **2015,** *13* (6), 343-359.
12. Lertpiriyapong, K.; Gamazon, E. R.; Feng, Y.; Park, D. S.; Pang, J.; Botka, G.; Graffam, M. E.; Ge, Z. M.; Fox, J. G., Campylobacter jejuni Type VI Secretion System: Roles in Adaptation to Deoxycholic Acid, Host Cell Adherence, Invasion, and In Vivo Colonization. *Plos One* **2012,** *7* (8).
13. Hancock, R. E. W.; Nikaido, H., Outer Membranes of Gram-Negative Bacteria .19. Isolation from Pseudomonas-Aeruginosa Pao1 and Use in Reconstitution and Definition of Permeability Barrier. *J Bacteriol* **1978,** *136* (1), 381-390.
14. Diedrich, D. L.; Cota-Robles, E. H., Heterogeneity in lipid composition of the outer membrane and cytoplasmic membrane and cytoplasmic membrane of Pseudomonas BAL-31. *J Bacteriol* **1974,** *119* (3), 1006-18.
15. Santarella-Mellwig, R.; Pruggnaller, S.; Roos, N.; Mattaj, I. W.; Devos, D. P., Three-dimensional reconstruction of bacteria with a complex endomembrane system. *PLoS biology* **2013,** *11* (5), e1001565.
16. Silhavy, T. J.; Kahne, D.; Walker, S., The bacterial cell envelope. *Cold Spring Harbor perspectives in biology* **2010,** *2* (5), a000414.
17. Perez-Cruz, C.; Carrion, O.; Delgado, L.; Martinez, G.; Lopez-Iglesias, C.; Mercade, E., New type of outer membrane vesicle produced by the Gram-negative bacterium Shewanella vesiculosa M7T: implications for DNA content. *Applied and environmental microbiology* **2013,** *79* (6), 1874-81.
18. Chatterjee, S. N.; Das, J., Electron microscopic observations on the excretion of cell-wall material by Vibrio cholerae. *Journal of general microbiology* **1967,** *49* (1), 1-11.
19. Kulkarni, H. M.; Jagannadham, M. V., Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiol-Sgm* **2014,** *160*, 2109-2121.

20. Chatterjee, D.; Chaudhuri, K., Association of cholera toxin with Vibrio cholerae outer membrane vesicles which are internalized by human intestinal epithelial cells. *Febs Lett* **2011,** *585* (9), 1357-1362.
21. Kulp, A.; Kuehn, M. J., Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annual Review of Microbiology, Vol 64, 2010* **2010,** *64*, 163-184.
22. Deatherage, B. L.; Cookson, B. T., Membrane Vesicle Release in Bacteria, Eukaryotes, and Archaea: a Conserved yet Underappreciated Aspect of Microbial Life. *Infect Immun* **2012,** *80* (6), 1948-1957.
23. Chattopadhyay, M. K.; Jagannadham, M. V., Vesicles-mediated resistance to antibiotics in bacteria (vol 6, 758, 2015). *Front Microbiol* **2015,** *6*.
24. Choi, D. S.; Kim, D. K.; Choi, S. J.; Lee, J.; Choi, J. P.; Rho, S.; Park, S. H.; Kim, Y. K.; Hwang, D.; Gho, Y. S., Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. *Proteomics* **2011,** *11* (16), 3424-3429.
25. Kaparakis-Liaskos, M.; Ferrero, R. L., Immune modulation by bacterial outer membrane vesicles. *Nature Reviews Immunology* **2015,** *15* (6), 375-387.
26. Veith, P. D.; Chen, Y. Y.; Gorasia, D. G.; Chen, D.; Glew, M. D.; O'Brien-Simpson, N. M.; Cecil, J. D.; Holden, J. A.; Reynolds, E. C., Porphyromonas gingivalis Outer Membrane Vesicles Exclusively Contain Outer Membrane and Periplasmic Proteins and Carry a Cargo Enriched with Virulence Factors. *Journal of proteome research* **2014,** *13* (5), 2420-2432.
27. Kouokam, J. C.; Wai, S. N.; Fallman, M.; Dobrindt, U.; Hacker, J.; Uhlin, B. E., Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic Escherichia coli. *Infect Immun* **2006,** *74* (4), 2022-2030.
28. Renelli, M.; Matias, V.; Lo, R. Y.; Beveridge, T. J., DNA-containing membrane vesicles of Pseudomonas aeruginosa PAO1 and their genetic transformation potential. *Microbiol-Sgm* **2004,** *150*, 2161-2169.
29. Ghosal, A.; Upadhyaya, B. B.; Fritz, J. V.; Heintz-Buschart, A.; Desai, M. S.; Yusuf, D.; Huang, D.; Baumuratov, A.; Wang, K.; Galas, D.; Wilmes, P., The extracellular RNA complement of Escherichia coli. *Microbiologyopen* **2015,** *4* (2), 252-266.
30. Schwechheimer, C.; Kuehn, M. J., Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* **2015,** *13* (10), 605-619.
31. (a) Schwechheimer, C.; Rodriguez, D. L.; Kuehn, M. J., NlpI-mediated modulation of outer membrane vesicle production through peptidoglycan dynamics in Escherichia coli. *Microbiologyopen* **2015,** *4* (3), 375-389; (b) Perez-Cruz, C.; Delgado, L.; Lopez-Iglesias, C.; Mercade, E., Outer-Inner Membrane Vesicles Naturally Secreted by Gram-Negative Pathogenic Bacteria. *Plos One* **2015,** *10* (1).
32. Kadurugamuwa, J. L.; Beveridge, T. J., Virulence Factors Are Released from Pseudomonas-Aeruginosa in Association with Membrane-Vesicles during Normal Growth and Exposure to Gentamicin - a Novel Mechanism of Enzyme-Secretion. *J Bacteriol* **1995,** *177* (14), 3998-4008.
33. Mashburn-Warren, L.; Howe, J.; Garidel, P.; Richter, W.; Steiniger, F.; Roessle, M.; Brandenburg, K.; Whiteley, M., Interaction of quorum signals with outer membrane lipids: insights into prokaryotic membrane vesicle formation. *Mol Microbiol* **2008,** *69* (2), 491-502.
34. Maldonado, R.; Wei, R.; Kachlany, S. C.; Kazi, M.; Balashova, N. V., Cytotoxic effects of Kingella kingae outer membrane vesicles on human cells. *Microb Pathogenesis* **2011,** *51* (1-2), 22-30.
35. George, D. T.; Behm, C. A.; Hall, D. H.; Mathesius, U.; Rug, M.; Nguyen, K. C. Q.; Verma, N. K., Shigella flexneri Infection in Caenorhabditis elegans: Cytopathological Examination and Identification of Host Responses. *Plos One* **2014,** *9* (9).
36. Kesty, N. C.; Kuehn, M. J., Incorporation of heterologous outer membrane and periplasmic proteins into Escherichia coli outer membrane vesicles. *Journal of Biological Chemistry* **2004,** *279* (3), 2069-2076.
37. Rompikuntal, P. K.; Thay, B.; Khan, M. K.; Alanko, J.; Penttinen, A. M.; Asikainen, S.; Wai, S. N.; Oscarsson, J., Perinuclear Localization of Internalized Outer Membrane Vesicles Carrying Active

Cytolethal Distending Toxin from Aggregatibacter actinomycetemcomitans. *Infect Immun* **2012,** *80* (1), 31-42.
38. Kolling, G. L.; Matthews, K. R., Export of virulence genes and shiga toxin by membrane vesicles of Escherichia coli O157 : H7 (vol 65, pg 1843, 1999). *Applied and environmental microbiology* **1999,** *65* (8), 3761-3761.
39. Parker, H.; Chitcholtan, K.; Hampton, M. B.; Keenan, J. I., Uptake of Helicobacter pylori Outer Membrane Vesicles by Gastric Epithelial Cells. *Infect Immun* **2010,** *78* (12), 5054-5061.
40. Wai, S. N.; Lindmark, B.; Soderblom, T.; Takade, A.; Westermark, M.; Oscarsson, J.; Jass, J.; Richter-Dahlfors, A.; Mizunoe, Y.; Uhlin, B. E., Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell* **2003,** *115* (1), 25-35.
41. Hozbor, D.; Rodriguez, M. E.; Fernandez, J.; Lagares, A.; Guiso, N.; Yantorno, O., Release of outer membrane vesicles from Bordetella pertussis. *Current microbiology* **1999,** *38* (5), 273-278.
42. Guo, L. N.; Fatig, R. O.; Orr, G. L.; Schafer, B. W.; Strickland, J. A.; Sukhapinda, K.; Woodsworth, A. T.; Petell, J. K., Photorhabdus luminescens W-14 insecticidal activity consists of at least two similar but distinct proteins - Purification and characterization of toxin A and toxin B. *Journal of Biological Chemistry* **1999,** *274* (14), 9836-9842.
43. Negrete-Abascal, E.; Garcia, R. M.; Reyes, M. E.; Godinez, D.; de la Garza, M., Membrane vesicles released by Actinobacillus pleuropneumoniae contain proteases and Apx toxins. *Fems Microbiol Lett* **2000,** *191* (1), 109-113.
44. Hellman, J.; Loiselle, P. M.; Tehan, M. M.; Allaire, J. E.; Boyle, L. A.; Kurnick, J. T.; Andrews, D. M.; Kim, K. S.; Warren, H. S., Outer membrane protein A, peptidoglycan-associated lipoprotein, and murein lipoprotein are released by Escherichia coli bacteria into serum. *Infect Immun* **2000,** *68* (5), 2566-2572.
45. Patrick, S.; McKenna, J. P.; OHagan, S.; Dermott, E., A comparison of the haemagglutinating and enzymic activities of Bacteroides fragilis whole cells and outer membrane vesicles. *Microb Pathogenesis* **1996,** *20* (4), 191-202.
46. Forsberg, C. W.; Beveridge, T. J.; Hellstrom, A., Cellulase and Xylanase Release from Bacteroides-Succinogenes and Its Importance in the Rumen Environment. *Applied and environmental microbiology* **1981,** *42* (5), 886-896.
47. Ciofu, O.; Beveridge, T. J.; Kadurugamuwa, J.; Walther-Rasmussen, J.; Hoiby, N., Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. *J Antimicrob Chemoth* **2000,** *45* (1), 9-13.
48. Bergman, M. A.; Cummings, L. A.; Barrett, S. L. R.; Smith, K. D.; Lara, J. C.; Aderem, A.; Cookson, B. T., CD4(+) T cells and toll-like receptors recognize Salmonella antigens expressed in bacterial surface organelles. *Infect Immun* **2005,** *73* (3), 1350-1356.
49. Kadurugamuwa, J. L.; Beveridge, T. J., Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using Shigella flexneri membrane vesicles. *Antimicrobial agents and chemotherapy* **1998,** *42* (6), 1476-1483.
50. Chi, B.; Qi, M.; Kuramitsu, H. K., Role of dentilisin in Treponema denticola epithelial cell layer penetration. *Research in microbiology* **2003,** *154* (9), 637-643.
51. Hong, G. E.; Kim, D. G.; Park, E. M.; Nam, B. H.; Kim, Y. O.; Kong, I. S., Identification of Vibrio anguillarum Outer Membrane Vesicles Related to Immunostimulation in the Japanese Flounder, Paralichthys olivaceus. *Biosci Biotech Bioch* **2009,** *73* (2), 437-439.
52. Manning, A. J.; Kuehn, M. J., Functional Advantages Conferred by Extracellular Prokaryotic Membrane Vesicles. *Journal of molecular microbiology and biotechnology* **2013,** *23* (1-2), 131-141.
53. Schertzer, J. W.; Whiteley, M., Bacterial Outer Membrane Vesicles in Trafficking, Communication and the Host-Pathogen Interaction. *J Mol Microb Biotech* **2013,** *23* (1-2), 118-130.
54. Schooling, S. R.; Beveridge, T. J., Membrane vesicles: an overlooked component of the matrices of biofilms. *J Bacteriol* **2006,** *188* (16), 5945-5957.

55. Muraca, M.; Putignani, L.; Fierabracci, A.; Teti, A.; Perilongo, G., Gut microbiota-derived outer membrane vesicles: under-recognized major players in health and disease? *Discov Med* **2015,** *19* (106), 343-8.
56. Kolling, G. L.; Matthews, K. R., Export of virulence genes and shiga toxin by membrane vesicles of Escherichia coli O157 : H7. *Applied and environmental microbiology* **1999,** *65* (5), 1843-1848.
57. Cluss, R. G.; Silverman, D. A.; Stafford, T. R., Extracellular secretion of the Borrelia burgdorferi Oms28 porin and Bgp, a glycosaminoglycan binding protein. *Infect Immun* **2004,** *72* (11), 6279-86.
58. Perry, R. D.; Fetherston, J. D., Yersinia pestis - Etiologic agent of plague. *Clinical microbiology reviews* **1997,** *10* (1), 35-&.
59. Eisen, R. J.; Bearden, S. W.; Wilder, A. P.; Montenieri, J. A.; Antolin, M. F.; Gage, K. L., Early-phase transmission of Yersinia pestis by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. *P Natl Acad Sci USA* **2006,** *103* (42), 15380-15385.
60. Fukushima, H.; Shimizu, S.; Inatsu, Y., Yersinia enterocolitica and Yersinia pseudotuberculosis Detection in Foods. *Journal of pathogens* **2011,** *2011*, 735308.
61. Fukushima, H.; Gomyoda, M.; Shiozawa, K.; Kaneko, S.; Tsubokura, M., Yersinia-Pseudotuberculosis Infection Contracted through Water Contaminated by a Wild Animal. *Journal of clinical microbiology* **1988,** *26* (3), 584-585.
62. Fukushima, H.; Hoshina, K.; Nakamura, R.; Ito, Y., Occurrence of Yersinia Spp in Raw Beef, Pork and Chicken. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Serie B-Umwelthygiene Krankenhaushygiene Arbeitshygiene Praventive Medizin* **1987,** *184* (1), 50-59.
63. (a) Fukushima, H., Direct Isolation of Yersinia-Pseudotuberculosis from Fresh-Water in Japan. *Appl Environ Microb* **1992,** *58* (8), 2688-2690; (b) Inoue, M.; Nakashima, H.; Ishida, T.; Tsubokura, M.; Sakazaki, R., Isolation of Yersinia Pseudotuberculosis from Water. *Zbl Hyg Umweltmed* **1988,** *186* (4), 338-343.
64. (a) Jalava, K.; Hallanvuo, S.; Nakari, U. M.; Ruutu, P.; Kela, E.; Heinasmaki, T.; Siitonen, A.; Nuorti, J. P., Multiple outbreaks of Yersinia pseudotuberculosis infections in Finland. *J Clin Microbiol* **2004,** *42* (6), 2789-2791; (b) Hayashidani, H.; Kanzaki, N.; Kaneko, Y.; Okatani, A. T.; Taniguchi, T.; Kaneko, K.; Ogawa, M., Occurrence of Yersiniosis and Listeriosis in wild boars in Japan. *J Wildlife Dis* **2002,** *38* (1), 202-205.
65. Nuorti, J. P.; Niskanen, T.; Hallanvuo, S.; Mikkola, J.; Kela, E.; Hatakka, M.; Fredriksson-Ahomaa, M.; Lyytikainen, O.; Siitonen, A.; Korkeala, H.; Ruutu, P., A widespread outbreak of Yersinia pseudotuberculosis O : 3 infection from iceberg lettuce. *Journal of Infectious Diseases* **2004,** *189* (5), 766-774.
66. (a) Eitel, J.; Heise, T.; Thiesen, U.; Dersch, P., Cell invasion and IL-8 production pathways initiated by YadA of Yersinia pseudotuberculosis require common signalling molecules (FAK, c-Src, Ras) and distinct cell factors. *Cell Microbiol* **2005,** *7* (1), 63-77; (b) Leong, J. M.; Fournier, R. S.; Isberg, R. R., Identification of the Integrin Binding Domain of the Yersinia-Pseudotuberculosis Invasin Protein. *Embo J* **1990,** *9* (6), 1979-1989; (c) Tsang, T. M.; Wiese, J. S.; Felek, S.; Kronshage, M.; Krukonis, E. S., Ail Proteins of Yersinia pestis and Y-pseudotuberculosis Have Different Cell Binding and Invasion Activities. *Plos One* **2013,** *8* (12).
67. (a) Maia, J. M. L.; Monnazzi, L. G. S.; Medeiros, B. M. M., Role of Yersinia pseudotuberculosis outer proteins (Yops) in murine humoral immune response. *Folia Microbiol* **2009,** *54* (3), 239-245; (b) Trosky, J. E.; Liverman, A. D. B.; Orth, K., Yersinia outer proteins: Yops. *Cell Microbiol* **2008,** *10* (3), 557-565; (c) Leary, S. E. C.; Griffin, K. F.; Galyov, E. E.; Hewer, J.; Williamson, E. D.; Holmstrom, A.; Forsberg, A.; Titball, R. W., Yersinia outer proteins (YOPS) E, K and N are antigenic but non-protective compared to V antigen, in a murine model of bubonic plague. *Microb Pathogenesis* **1999,** *26* (3), 159-169.
68. (a) Cornelis, G. R.; WolfWatz, H., The Yersinia Yop virulon: A bacterial system for subverting eukaryotic cells. *Mol Microbiol* **1997,** *23* (5), 861-867; (b) Cornelis, G. R.; Boland, A.; Boyd, A. P.; Geuijen, C.; Iriarte, M.; Neyt, C.; Sory, M. P.; Stainier, I., The virulence plasmid of Yersinia, an antihost genome. *Microbiol Mol Biol R* **1998,** *62* (4), 1315-+; (c) Cornelis, G. R., The Yersinia deadly kiss. *J Bacteriol* **1998,**

180 (21), 5495-5504; (d) Galan, J. E.; Wolf-Watz, H., Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **2006,** *444* (7119), 567-573.
69. Schweer, J.; Kulkarni, D.; Kochut, A.; Pezoldt, J.; Pisano, F.; Pils, M. C.; Genth, H.; Huehn, J.; Dersch, P., The Cytotoxic Necrotizing Factor of Yersinia pseudotuberculosis (CNFY) Enhances Inflammation and Yop Delivery during Infection by Activation of Rho GTPases. *Plos Pathog* **2013,** *9* (11).
70. Lockman, H. A.; Gillespie, R. A.; Baker, B. D.; Shakhnovich, E., Yersinia pseudotuberculosis produces a cytotoxic necrotizing factor. *Infect Immun* **2002,** *70* (5), 2708-2714.
71. Ellis, T. N.; Kuehn, M. J., Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles. *Microbiol Mol Biol R* **2010,** *74* (1), 81-+.
72. Nguyen, T. T.; Saxena, A.; Beveridge, T. J., Effect of surface lipopolysaccharide on the nature of membrane vesicles liberated from the Gram-negative bacterium Pseudomonas aeruginosa. *J Electron Microsc* **2003,** *52* (5), 465-469.
73. (a) Berleman, J.; Auer, M., The role of bacterial outer membrane vesicles for intra- and interspecies delivery. *Environ Microbiol* **2013,** *15* (2), 347-354; (b) Kuehn, M. J.; Kesty, N. C., Bacterial outer membrane vesicles and the host-pathogen interaction. *Gene Dev* **2005,** *19* (22), 2645-2655.
74. Skurnik, M.; Peippo, A.; Ervela, E., Characterization of the O-antigen gene clusters of Yersinia pseudotuberculosis and the cryptic O-antigen gene cluster of Yersinia pestis shows that the plague bacillus is most closely related to and has evolved from Y-pseudotuberculosis serotype O : 1b. *Mol Microbiol* **2000,** *37* (2), 316-330.
75. Fukushima, H.; Gomyoda, M.; Ishikura, S.; Nishio, T.; Moriki, S.; Endo, J.; Kaneko, S.; Tsubokura, M., Cat-Contaminated Environmental Substances Lead to Yersinia-Pseudotuberculosis Infection in Children. *J Clin Microbiol* **1989,** *27* (12), 2706-2709.
76. Marra, A.; Isberg, R. R., Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium. *Infect Immun* **1997,** *65* (8), 3412-3421.
77. Carniel, E.; Mollaret, H. H., Yersiniosis. *Comp Immunol Microb* **1990,** *13* (2), 51-58.
78. Jelloul, L.; Fremond, B.; Dyon, J. F.; Orme, R. L. E.; Babut, J. M., Mesenteric adenitis caused by Yersinia pseudotuberculosis presenting as an abdominal mass. *Eur J Pediatr Surg* **1997,** *7* (3), 180-183.
79. Brubaker, R. R., Factors Promoting Acute and Chronic Diseases Caused by Yersiniae. *Clin Microbiol Rev* **1991,** *4* (3), 309-324.
80. Sanford, S. E., Outbreaks of Yersiniosis Caused by Yersinia-Pseudotuberculosis in Farmed Cervids. *J Vet Diagn Invest* **1995,** *7* (1), 78-81.
81. Thorslund, S. E.; Edgren, T.; Pettersson, J.; Nordfelth, R.; Sellin, M. E.; Ivanova, E.; Francis, M. S.; Isaksson, E. L.; Wolf-Watz, H.; Fallman, M., The RACK1 Signaling Scaffold Protein Selectively Interacts with Yersinia pseudotuberculosis Virulence Function. *Plos One* **2011,** *6* (2).
82. Viboud, G. I.; Bliska, J. B., Yersinia outer proteins: Role in modulation of host cell signaling responses and pathogenesis. *Annu Rev Microbiol* **2005,** *59*, 69-89.
83. Bomberger, J. M.; MacEachran, D. P.; Coutermarsh, B. A.; Ye, S. Y.; O'Toole, G. A.; Stanton, B. A., Long-Distance Delivery of Bacterial Virulence Factors by Pseudomonas aeruginosa Outer Membrane Vesicles. *Plos Pathog* **2009,** *5* (4).
84. Lee, C.; Kim, J.; Shin, S. G.; Hwang, S., Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. *Journal of biotechnology* **2006,** *123* (3), 273-80.
85. Shevchenko, A.; Tomas, H.; Havlis, J.; Olsen, J. V.; Mann, M., In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **2006,** *1* (6), 2856-2860.
86. Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R., A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* **2003,** *75* (17), 4646-4658.
87. Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T.; Harris, M. A.; Hill, D. P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J. C.; Richardson, J. E.; Ringwald, M.; Rubin, G. M.; Sherlock, G., Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics* **2000,** *25* (1), 25-9.

88. McCaig, W. D.; Koller, A.; Thanassi, D. G., Production of Outer Membrane Vesicles and Outer Membrane Tubes by Francisella novicida. *J Bacteriol* **2013,** *195* (6), 1120-1132.
89. Sotolongo, J.; Ruiz, J.; Fukata, M., The role of innate immunity in the host defense against intestinal bacterial pathogens. *Current infectious disease reports* **2012,** *14* (1), 15-23.
90. Bieber, K.; Autenrieth, S. E., Insights how monocytes and dendritic cells contribute and regulate immune defense against microbial pathogens. *Immunobiology* **2015,** *220* (2), 215-26.
91. Gong, L.; Devenish, R. J.; Prescott, M., Autophagy as a macrophage response to bacterial infection. *Iubmb Life* **2012,** *64* (9), 740-747.
92. Chang, S. C.; Ding, J. L., Ubiquitination by SAG regulates macrophage survival/death and immune response during infection. *Cell Death and Differentiation* **2014,** *21* (9), 1388-1398.
93. Costa, T. R.; Felisberto-Rodrigues, C.; Meir, A.; Prevost, M. S.; Redzej, A.; Trokter, M.; Waksman, G., Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **2015,** *13* (6), 343-59.
94. Beveridge, T. J., Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol* **1999,** *181* (16), 4725-4733.
95. Ellis, T. N.; Leiman, S. A.; Kuehn, M. J., Naturally Produced Outer Membrane Vesicles from Pseudomonas aeruginosa Elicit a Potent Innate Immune Response via Combined Sensing of Both Lipopolysaccharide and Protein Components. *Infect Immun* **2010,** *78* (9), 3822-3831.
96. Meng, F. Y.; Lowell, C. A., Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *Journal of Experimental Medicine* **1997,** *185* (9), 1661-1670.
97. Guthrie, T.; Wong, S. Y.; Liang, B.; Hyland, L.; Hou, S.; Hoiby, E. A.; Andersen, S. R., Local and systemic antibody responses in mice immunized intranasally with native and detergent-extracted outer membrane vesicles from Neisseria meningitidis. *Infect Immun* **2004,** *72* (5), 2528-37.
98. Schild, S.; Nelson, E. J.; Camilli, A., Immunization with Vibrio cholerae outer membrane vesicles induces protective immunity in mice. *Infect Immun* **2008,** *76* (10), 4554-63.
99. Winter, J.; Letley, D.; Rhead, J.; Atherton, J.; Robinson, K., Helicobacter pylori membrane vesicles stimulate innate pro- and anti-inflammatory responses and induce apoptosis in Jurkat T cells. *Infect Immun* **2014,** *82* (4), 1372-81.
100. Schaar, V.; de Vries, S. P.; Perez Vidakovics, M. L.; Bootsma, H. J.; Larsson, L.; Hermans, P. W.; Bjartell, A.; Morgelin, M.; Riesbeck, K., Multicomponent Moraxella catarrhalis outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells. *Cell Microbiol* **2011,** *13* (3), 432-49.
101. Bielig, H.; Dongre, M.; Zurek, B.; Wai, S. N.; Kufer, T. A., A role for quorum sensing in regulating innate immune responses mediated by Vibrio cholerae outer membrane vesicles (MVSs). *Gut microbes* **2011,** *2* (5), 274-9.
102. (a) Tauxe, R. V., Salad and pseudoappendicitis: Yersinia pseudotuberculosis as a foodborne pathogen. *Journal of Infectious Diseases* **2004,** *189* (5), 761-763; (b) Westermark, L.; Fahlgren, A.; Fallman, M., Yersinia pseudotuberculosis Efficiently Escapes Polymorphonuclear Neutrophils during Early Infection. *Infect Immun* **2014,** *82* (3), 1181-1191.
103. Pujol, C.; Bliska, J. B., The ability to replicate in macrophages is conserved between Yersinia pestis and Yersinia pseudotuberculosis. *Infect Immun* **2003,** *71* (10), 5892-5899.
104. Ramana, K. V.; Reddy, A. B. M.; Tammali, R.; Srivastava, S. K., Aldose reductase mediates endotoxin-induced production of nitric oxide and cytotoxicity in murine macrophages. *Free Radical Biology and Medicine* **2007,** *42* (8), 1290-1302.
105. Zhang, Y.; Murtha, J.; Roberts, M. A.; Siegel, R. M.; Bliska, J. B., Type III secretion decreases bacterial and host survival following phagocytosis of Yersinia pseudotuberculosis by macrophages. *Infect Immun* **2008,** *76* (9), 4299-4310.
106. Parent, M. A.; Wilhelm, L. B.; Kummer, L. W.; Szaba, F. M.; Mullarky, I. K.; Smiley, S. T., Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity,

perform critical protective functions during humoral defense against lethal pulmonary Yersinia pestis infection. *Infect Immun* **2006,** *74* (6), 3381-3386.
107. Martin, H. R.; Taylor, N. S.; Buckley, E. M.; Marini, R. P.; Patterson, M. M.; Fox, J. G., Characterization of cytotoxic necrotizing factor 1-producing Escherichia coli strains from faeces of healthy macaques. *Journal of medical microbiology* **2009,** *58* (10), 1354-1358.
108. Blumenthal, B.; Hoffmann, C.; Aktories, K.; Backert, S.; Schmidt, G., The cytotoxic necrotizing factors from Yersinia pseudotuberculosis and from Escherichia coli bind to different cellular receptors but take the same route to the cytosol. *Infect Immun* **2007,** *75* (7), 3344-53.
110. Lemonnier, M.; Landraud, L.; Lemichez, E., Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. *Fems Microbiol Rev* **2007,** *31* (5), 515-534.
111. Piteau, M.; Papatheodorou, P.; Schwan, C.; Schlosser, A.; Aktories, K.; Schmidt, G., Lu/BCAM Adhesion Glycoprotein Is a Receptor for Escherichia coli Cytotoxic Necrotizing Factor 1 (CNF1). *Plos Pathog* **2014,** *10* (1).
112. Fabbri, A.; Travaglione, S.; Fiorentini, C., Escherichia coli Cytotoxic Necrotizing Factor 1 (CNF1): Toxin Biology, in Vivo Applications and Therapeutic Potential. *Toxins* **2010,** *2* (2), 283-296.
113. Buetow, L.; Flatau, G.; Chiu, K.; Boquet, P.; Ghosh, P., Structure of the Rho-activating domain of Escherichia coli cytotoxic necrotizing factor 1. *Nat Struct Biol* **2001,** *8* (7), 584-588.
114. Wang, Y. C.; Peterson, S. E.; Loring, J. F., Protein post-translational modifications and regulation of pluripotency in human stem cells. *Cell Res* **2014,** *24* (2), 143-160.
115. Karve, T. M.; Cheema, A. K., Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease. *Journal of amino acids* **2011,** *2011*, 207691.
116. Choudhary, C.; Weinert, B. T.; Nishida, Y.; Verdin, E.; Mann, M., The growing landscape of lysine acetylation links metabolism and cell signalling. *Nature reviews. Molecular cell biology* **2014,** *15* (8), 536-50.
117. Ribet, D.; Cossart, P., Post-translational modifications in host cells during bacterial infection. *Febs Lett* **2010,** *584* (13), 2748-58.
118. Walsh, G.; Jefferis, R., Post-translational modifications in the context of therapeutic proteins. *Nat Biotechnol* **2006,** *24* (10), 1241-52.
119. Szymanski, C. M.; Burr, D. H.; Guerry, P., Campylobacter protein glycosylation affects host cell interactions. *Infect Immun* **2002,** *70* (4), 2242-4.
120. Beausoleil, S. A.; Villen, J.; Gerber, S. A.; Rush, J.; Gygi, S. P., A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol* **2006,** *24* (10), 1285-1292.
121. Bernaudat, F.; Frelet-Barrand, A.; Rochon, N.; Dementin, S.; Hivin, P.; Boutigny, S.; Rioux, J. B.; Salvi, D.; Seigneurin-Berny, D.; Richaud, P.; Joyard, J.; Pignol, D.; Sabaty, M.; Desnos, T.; Pebay-Peyroula, E.; Darrouzet, E.; Vernet, T.; Rolland, N., Heterologous expression of membrane proteins: choosing the appropriate host. *PLoS One* **2011,** *6* (12), e29191.
122. Zhang, X.; Wen, H.; Shi, X. B., Lysine methylation: beyond histones. *Acta Bioch Bioph Sin* **2012,** *44* (1), 14-27.
123. Cao, X. J.; Dai, J.; Xu, H.; Nie, S.; Chang, X.; Hu, B. Y.; Sheng, Q. H.; Wang, L. S.; Ning, Z. B.; Li, Y. X.; Guo, X. K.; Zhao, G. P.; Zeng, R., High-coverage proteome analysis reveals the first insight of protein modification systems in the pathogenic spirochete Leptospira interrogans. *Cell Res* **2010,** *20* (2), 197-210.
124. Abeykoon, A. H.; Chao, C. C.; Wang, G. H.; Gucek, M.; Yang, D. C. H.; Ching, W. M., Two Protein Lysine Methyltransferases Methylate Outer Membrane Protein B from Rickettsia. *J Bacteriol* **2012,** *194* (23), 6410-6418.
125. Garbom, S.; Forsberg, A.; Wolf-Watz, H.; Kihlberg, B. M., Identification of novel virulence-associated genes via genome analysis of hypothetical genes. *Infect Immun* **2004,** *72* (3), 1333-1340.
126. Sherris, D.; Parkinson, J. S., Posttranslational Processing of Methyl-Accepting Chemotaxis Proteins in Escherichia-Coli. *P Natl Acad Sci-Biol* **1981,** *78* (10), 6051-6055.

127. Sprung, R.; Chen, Y.; Zhang, K.; Cheng, D.; Zhang, T.; Peng, J.; Zhao, Y., Identification and validation of eukaryotic aspartate and glutamate methylation in proteins. *Journal of proteome research* **2008,** *7* (3), 1001-1006.
128. Choudhary, C.; Weinert, B. T.; Nishida, Y.; Verdin, E.; Mann, M., The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat Rev Mol Cell Bio* **2014,** *15* (8), 536-550.
129. Ishfaq, M.; Maeta, K.; Maeda, S.; Natsume, T.; Ito, A.; Yoshida, M., Acetylation regulates subcellular localization of eukaryotic translation initiation factor 5A (eIF5A) (vol 586, pg 3236, 2012). *Febs Lett* **2014,** *588* (16), 2754-2754.
130. Li, X.; Lin, C.; O'Connor, P. B., Glutamine deamidation: differentiation of glutamic acid and gamma-glutamic acid in peptides by electron capture dissociation. *Anal Chem* **2010,** *82* (9), 3606-15.
131. Robinson, A. B.; Rudd, C. J., Deamidation of glutaminyl and asparaginyl residues in peptides and proteins. *Current topics in cellular regulation* **1974,** *8* (0), 247-95.